Laboratory of Analytical Chemistry
Department of Chemistry
University of Helsinki
Finland

STABILIZATION OF PHOSPHOLIPID COATINGS IN CAPILLARY ELECTROPHORESIS

Maria Viola Lindén

Academic Dissertation

To be presented, with permission of
the Faculty of Science of the University of Helsinki,
for public criticism in Chemicum, Auditorium A 129,
(A.I.Virtasen aukio 1, Helsinki)
on October 24, at 12 o'clock noon.

Helsinki 2008

Supervisor:
Docent Susanne Wiedmer
Laboratory of Analytical Chemistry
Department of Chemistry
Faculty of Science
University of Helsinki
Finland

Reviewers:
Professor Ernst Kenndler
Institute of Analytical Chemistry
University of Vienna
Austria

and

Professor J. Peter Slotte
Laboratory of Lipid and Membrane Biochemistry
Department of Biochemistry and Pharmacy
Faculty of Mathematics and Natural Sciences
Åbo Akademi University
Finland

Opponent:
Professor Katarina Edwards
Department of Physical and Analytical Chemistry
Faculty of Science and Technology
Uppsala University
Sweden

Custos:
Professor Marja-Liisa Riekkola
Laboratory of Analytical Chemistry
Department of Chemistry
Faculty of Science
University of Helsinki
Finland

ISBN 978-952-92-4189-7 (paperback)
ISBN 978-952-10-4841-8 (PDF)
http://ethesis.helsinki.fi
Helsinki University Printing House, Helsinki 2008

TABLE OF CONTENTS

PREFACE **5**

ABSTRACT **6**

ABBREVIATIONS **7**

SYMBOLS **8**

LIST OF ORIGINAL PUBLICATIONS **9**

1 INTRODUCTION AND AIMS **10**

2 PHOSPHOLIPIDS **12**
2.1 Liposomes **13**
2.2 Biological membranes **14**
2.3 Cholesterol in phospholipid bilayers **15**
2.4 Divalent cations in phospholipid bilayers **16**
2.5 Cetyltrimethylammonium bromide in phospholipid bilayers **17**
2.6 Polyethylene glycol-stabilized bilayers **18**

3 CAPILLARY ELECTROPHORESIS **19**

4 PHOSPHOLIPID-COATED CAPILLARIES **21**

5 EXPERIMENTAL **25**
5.1 Chemicals **25**
5.2 Sample and buffer preparation **25**
5.3 Liposome preparation **26**
5.4 Preparation of liposomes from red blood cell ghost lipids **26**
5.5 Instrumentation **27**
5.5.1 Capillary electrophoresis 27
5.5.2 Differential scanning calorimetry 28
5.5.3 Asymmetrical flow field flow fractionation 28
5.5.4 Dynamic light scattering 28
5.5.5 Electron microscopy 29
5.5.6 Atomic force microscopy 29
5.5.7 Measurements with a quartz crystal microbalance 29

6 RESULTS AND DISCUSSION **31**
6.1 Development of the coating procedure **31**
6.1.1 Preconditioning of the capillary 31
6.1.2 Optimization of the phospholipid coating method 31
6.1.2.1 Coating time 31
6.1.2.2 Phospholipid concentration 32
6.1.3 Conditions for capillary storage 34
6.2 Stabilization of the phospholipid coating **34**

6.2.1 Stability of phosphatidylcholine coatings 35
6.2.2 Addition of divalent cations 37
6.2.3 Addition of cetyltrimethylammonium bromide 42
6.2.4 Adjustment of temperature 44
6.2.4.1 Differential scanning calorimetric studies 44
6.2.4.2 Coating and running temperatures 47
6.2.5 Addition of cholesterol 49
6.2.6 Addition of polyethylene glycol phospholipids 52
6.2.6.1 Sizes and zeta potentials 52
6.2.6.2 Aggregates as capillary coating material 53
6.2.7 Addition of cetyltrimethylammonium bromide in the coating 58
6.3 Human red blood cell ghost lipid coating 59

7 CONCLUSIONS 62

8 REFERENCES 64

APPENDIXES: PAPERS I-IV

PREFACE

This thesis is based on experimental work carried out in the Laboratory of Analytical Chemistry at the Department of Chemistry, University of Helsinki, during the years 2003-2008. Funding was provided by the Academy of Finland.

I am most grateful to my supervisor, Docent Susanne Wiedmer for her help and encouragement and the time she so generously invested. I would like to thank the head of the laboratory, Prof. Marja-Liisa Riekkola, for giving me the opportunity to carry out research in the Laboratory of Analytical Chemistry and Prof. Markku Räsänen for arranging the facilities for me to write the final manuscript.

Special thanks go to former and present laboratory staff and co-workers for their support and for creating a pleasant working atmosphere. My project colleague Jari Hautala deserves special mention for his kindness and patience during the years we worked together. Important help was also received from my other co-authors, especially Ruth Shimmo (formerly Kuldvee) and Juha Holopainen.

I would like to thank Pentti Jyske and Matti Jussila for solving various instrumental and computer problems, Merit Hortling for taking care of the paper work and for engaging discussions, and Liisa Heino for providing the chemicals, glassware, and supplies needed in the research. I am indebted to Kathleen Ahonen for improving the language of the dissertation and the original papers.

Finally, my warmest thanks go to my loving and caring family: my parents Anita and Ralf, my brother Benjamin, Tove, my adorable nieces Pernilla and Mathilda, and my other relatives and godmothers. You are all very dear to me. I am also grateful to my friends for their care and support and for innumerable dinners, walks, and discussions. Above all, I am grateful to my mum for her endless love and for encouraging me to persist in my endeavors through the downs as well as ups of life. Tack mamma!

ABSTRACT

The development of a simple method of coating a semi-permanent phospholipid layer onto a capillary for electrochromatography use was the focus of this study. The work involved finding good coating conditions, stabilizing the phospholipid coating, and examining the effect of adding divalent cations, cetyltrimethylammonium bromide, and polyethylene glycol (PEG)-lipids on the stability of the coating. Since a further purpose was to move toward more biological membrane coatings, the capillaries were also coated with cholesterol-containing liposomes and liposomes of red blood cell ghost lipids.

Liposomes were prepared by extrusion, and large unilamellar vesicles with a diameter of about 100 nm were obtained. Zwitterionic phosphatidylcholine (PC) was used as a basic component, mainly 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) but also eggPC and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). Different amounts of sphingomyelin, bovine brain phosphatidylserine, and cholesterol were added to the PC. The stability of the coating in 40 mM *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES) solution at pH 7.4 was studied by measuring the electroosmotic flow and by separating neutral steroids, basic proteins, and low-molar-mass drugs.

The presence of PC in the coating solution was found to be essential to achieving a coating. The stability of the coating was improved by the addition of negative phosphatidylserine, cholesterol, divalent cations, or PEGylated lipids, and by working in the gel-state region of the phospholipid. Study of the effect on the PC coating of divalent metal ions calcium, magnesium, and zinc showed a molar ratio of 1:3 PC/Ca^{2+} or PC/Mg^{2+} to give increased rigidity to the membrane and the best coating stability.

The PEGylated lipids used in the study were sterically stabilized commercial lipids with covalently attached PEG chains. The vesicle size generally decreased when PEGylated lipids of higher molar mass were present in the vesicle. The predominance of discoidal micelles over liposomes increased PEG chain length and the average size of the vesicles thus decreased. In the capillary electrophoresis (CE) measurements a highly stable electroosmotic flow was achieved with 20% PEGylated lipid in the POPC coating dispersion, the best results being obtained for disteroyl PEG (3000) conjugates. The results suggest that smaller particles (discoidal micelles) result in tighter packing and better shielding of silanol groups on the silica wall.

The effect of temperature on the coating stability was investigated by using DPPC liposomes at temperatures above (45 °C) and below (25 °C) the main phase transition temperature. Better results were obtained with DPPC in the more rigid gel state than in the fluid state: the electroosmotic flow was heavily suppressed and the PC coating was stabilized. Also dispersions of DPPC with 0–30 mol% of cholesterol and sphingomyelin in different ratios, which more closely resemble natural membranes, resulted in stable coatings. Finally, the CE measurements revealed that a stable coating is formed when capillaries are coated with liposomes of red blood cell ghost lipids.

ABBREVIATIONS

AFM	Atomic force microscopy
AsFlFFF	Asymmetrical flow field-flow fractionation
BGE	Background electrolyte
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
chol	Cholesterol
CTAB	Cetyltrimethylammonium bromide
DLS	Dynamic light scattering
DMPE-PEG	1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)]
DMPG	1,2-Dimyristoyl-*sn*-glycero-3-phosphoglycerol
DMSO	Dimethylsulfoxide
DOPC	1,2-Dioleyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DSC	Differential scanning calorimetry
DSPE-PEG	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)]
eggPC	Egg phosphatidylcholine
EOF	Electroosmotic flow
FITC	Fluorescein isothiocyanate
HEPES	*N*-(2-Hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid)
HSDSC	High-sensitivity differential scanning calorimetry
HV	High voltage
ID	Inner diameter
LEKC	Liposome electrokinetic chromatography
LUV	Large unilamellar vesicles
MeOH	Methanol
MLV	Multilamellar vesicles
OD	Outer diameter
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PS	Phosphatidylserine, 1,2-Diacyl-*sn*-glycero-3-phospho-L-serine
QCM	Quartz crystal microbalance
RBC	Red blood cell
RSD	Relative standard deviation
SM	Sphingomyelin, Ceramide-1-phosphocholine
SPB	Supported phospholipid bilayer
SVL	Supported vesicle layer
UV	Ultraviolet

SYMBOLS

C_p	Heat capacity [$kJmol^{-1}°C^{-1}$]
L_{det}	Capillary length to the detector [m]
L_{tot}	Total length of the capillary [m]
n	Number of runs
N	Plate number
pI	Isoelectric point
T	Temperature [°C]
T_m	Main phase transition temperature [°C]
t_m	Migration time [s]
U	Separation voltage [V]
w_h	Peak width at half height [s]
μ_{eo}	Electroosmotic mobility [$m^2V^{-1}s^{-1}$]
μ_{ep}	Electrophoretic mobility [$m^2V^{-1}s^{-1}$]
μ_{tot}	Total electrophoretic mobility [$m^2V^{-1}s^{-1}$]

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following five publications (in chronological order), hereafter referred to by their Roman numerals:

I **Simple coating of capillaries with anionic liposomes in capillary electrophoresis**
Hautala, J.T., Lindén, M.V., Wiedmer, S.K., Ryhänen, S.J., Säily, M.J., Kinnunen, P.K.J., Riekkola, M.-L., *Journal of Chromatography A 1004* (2003) 81-90. Copyright 2003 Elsevier Science

II **Stabilization of phosphatidylcholine coatings in capillary electrophoresis by increase in membrane rigidity**
Lindén, M.V., Wiedmer, S.K., Hakala, R.M.S, Riekkola, M.-L., *Journal of Chromatography A 1051* (2004) 61-68. Copyright 2004 Elsevier Science

III **Influence of cetyltrimethylammonium bromide on phosphatidylcholine-coated capillaries**
Kuldvee, R., Lindén, M.V., Wiedmer, S.K., Riekkola, M.-L., *Analytical and Bioanalytical Chemistry 380* (2004) 293-302. Copyright 2004 Springer-Verlag

IV **Cholesterol-rich membrane coatings for interaction studies in capillary electrophoresis: application to red blood cell lipid extracts**
Lindén, M.V., Holopainen, J., Laukkanen, A., Riekkola, M.-L., Wiedmer, S.K., *Electrophoresis 27* (2006) 3988-3998. Copyright 2006 Wiley-VCH

V **Characterization of phosphatidylcholine/polyethylene glycol–lipid aggregates and their use as coatings and carriers in capillary electrophoresis**
Lindén, M.V., Meinander, K., Helle, A., Yohannes, G., Riekkola, M.-L., Butcher, S.J., Viitala T., Wiedmer, S.K., *Electrophoresis 29* (2008) 852-862. Copyright 2008 Wiley-VCH

The author's contribution:
Paper I: The experimental work of sections 3.3, and (in part) writing of the article.
Papers II: The experimental work and writing of the article.
Paper III: Half of the experimental work and participation in writing of the article.
Papers IV: The experimental work and writing of the article.
Paper V: The experimental work of sections 3.3, 3.4, and 3.5 related to capillary electrochromatography and writing of the article.

1 INTRODUCTION AND AIMS

Capillary electrophoresis (CE) is an analytical separation technique in which analytes are separated in a liquid-filled capillary while voltage is applied. The analytes have a specific electrophoretic mobility in the electric field and, after being separated according to charge and size, are detected at the end of the capillary, usually by ultraviolet (UV) absorption. There are several sub-techniques in CE, such as capillary zone electrophoresis (CZE), capillary electrochromatography (CEC), and electrokinetic chromatography (EKC). In CZE proper, only charged analytes can be separated. In CEC and EKC, retention results from a combination of electrophoretic migration and a chromatographic distribution mechanism. CEC employs a packed or wall-coated capillary, whereas EKC employs a pseudo-stationary phase.

The focus of this study was a CEC method utilizing a semi-permanent phospholipid coating. A phospholipid-coated capillary was preferred to liposomes free in the BGE because phospholipids are of relatively high cost, and the amount needed to coat a capillary is much smaller than the amount needed for the pseudo-stationary phase. With the phospholipids attached to the capillary wall, it is also possible to connect the capillary to a mass spectrometer for the detection. The goal of this work was to develop a simple coating method and to test different phospholipid compositions and additives with a view to obtaining good stability of the coating. A further objective was to move toward more natural membrane coatings.

First, the preconditioning and coating method was optimized (paper I). Then the effect of divalent cations on the phospholipid coating stability and rigidity was investigated, and model basic proteins were separated on the coatings (paper II). The phospholipid coating shields the free silanols and allows separation of basic proteins, which would otherwise strongly interact with the silica wall. In the case of neutral steroids, the phospholipid coating also acts as a chromatographic phase. Model steroids were separated on phosphatidylcholine (PC) coatings (paper I) as well as on cholesterol-containing and human red blood cell ghost lipid coatings (paper IV). The influence of cetyltrimethylammonium bromide (CTAB) and Ca^{2+}-ions on PC coatings was investigated (paper III). In addition, the effect of temperature on the phospholipid phase was studied by CE (papers II-III) and by high sensitivity differential scanning calorimetry (HSDSC) (paper III).

In the final phase of the work (paper V), the aim was to determine the properties (size, surface charge, and hydrophobicity) of different polyethylene glycol (PEG)-lipid aggregates. The sizes of the vesicles were determined by light scattering, electron microscopy (EM), and asymmetrical flow field-flow fractionation (AsFlFFF), and the surface charges were measured by electrophoresis with a zetasizer. The packing of the PEGylated lipid aggregates on silica was studied by atomic force microscopy (AFM), EM, and CEC and with a quartz crystal microbalance (QCM). Separations of low-molar-mass analytes were studied to evaluate the shielding effect of the PEG-lipids against lipid–analyte interactions

Chapters 2–4 below provide an introduction to phospholipids, CE, and the use of phospholipids in CEC. Chapter 5 gives the experimental details. The results are presented and discussed in chapter 6, and chapter 7 summarizes the results and offers conclusions.

Research aims:

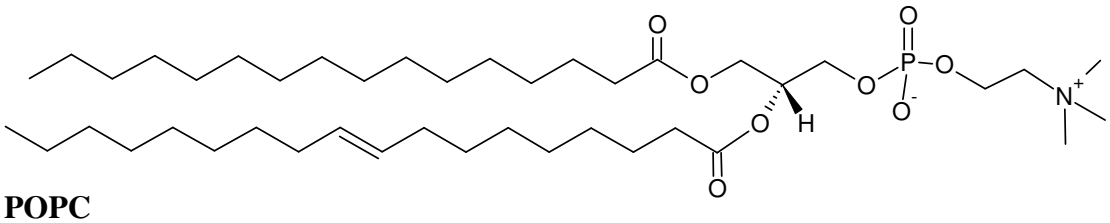
The main goal was to develop a stable phospholipid coating for CE that could be used for the study of analyte–membrane interactions. The ultimate aim was to move toward membranes that are more natural.

Specifically, the research aims were

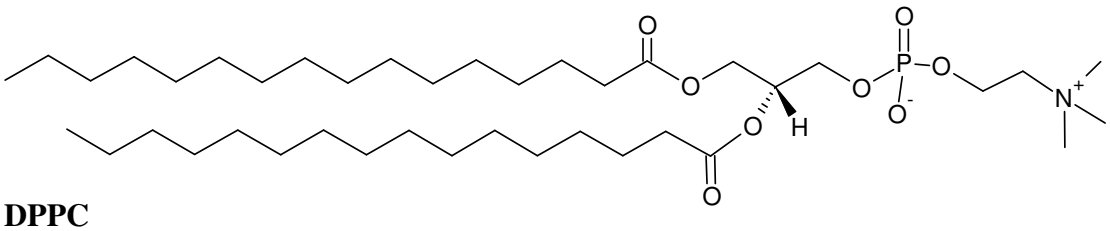
- to develop and optimize a method for coating phospholipids on CE capillaries (I)
- to improve the stability of the phospholipid coating through the addition of divalent cations (II)
- to improve the stability of the phospholipid coating through the addition of CTAB (III)
- to improve the stability of the phospholipid coating through the addition of PEGylated lipids and to study the characteristics of the coating (V)
- to examine the gel–fluid phase transition of the phospholipid membrane and its effect on the coating stability (IV)
- to move toward natural membrane coatings by using RBC ghost lipids in coating solutions (IV)

2 PHOSPHOLIPIDS

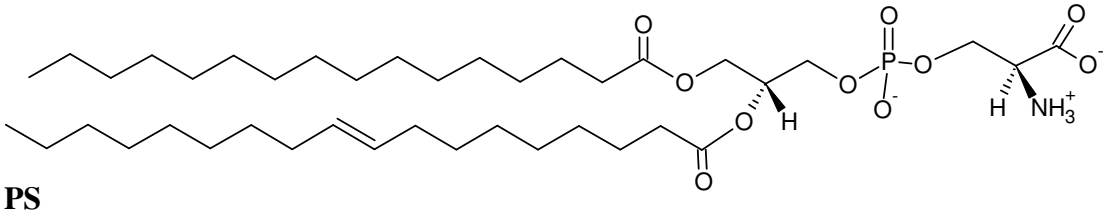
Phospholipids are the most widely present natural membrane lipids. The phospholipids are amphiphilic molecules with hydrophobic and hydrophilic domains. The head of a phospholipid is hydrophilic (attracted to water), while the hydrocarbon tails are hydrophobic. Phospholipids are of two types, glycerophospholipids and sphingophospholipids, which are derivatives of glycerol and sphingosine, respectively. The most common glycerophospholipid is zwitterionic phosphatidylcholine (PC), which can be considered to constitute the backbone of all cellular membranes. Figure 1 shows the structures of the phospholipids used in this study.



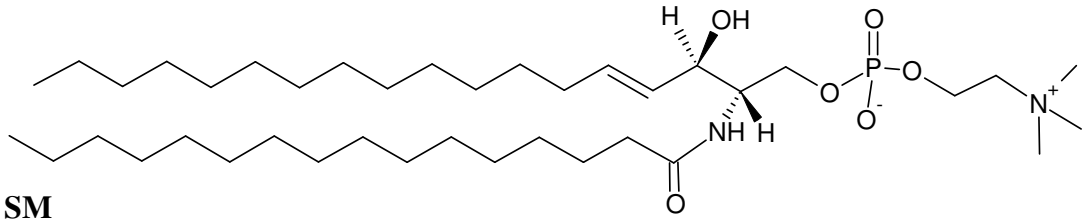
POPC
1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
16:0-18:1 PC



DPPC
1,2 Dipalmitoyl-*sn*-glycero-3-phosphocholine
16:0 PC



PS
1,2-Diacyl-*sn*-glycero-3-phospho-L-serine (bovine brain)
Fatty acid percentage: 18:0 42%; 18:1 34%; 20:6 8%; other 16%



SM
Sphingomyelin, ceramide-1-phosphocholine (chicken egg yolk)
Fatty acid percentage: 16:0 84%; 18:0 6%; other 10%

Figure 1. Molecular structures of phospholipids used in this work. Typical molecular structure is presented for PS and SM.

The glycerophospholipids and sphingomyelin are characterized by two fatty acid chains of various lengths and saturation. In the glycerophospholipids the fatty acid chains are linked via ester bonds to a glycerol backbone. The different headgroups are connected to the backbone via a phosphate group. The structure of sphingomyelin, with phosphorylcholine esterified to ceramide, is similar to that of phosphatidylcholine.

2.1 Liposomes

The amphiphilic nature of phospholipids causes them to self-assemble into lamellar bilayers in aqueous solution with spontaneous formation of phospholipid vesicles, or liposomes [1]. The thickness of a bilayer is ~ 4 nm and the diameter of the liposome can vary from nanometers to several micrometers. Many different methods have been applied to study the properties of liposomes [2] and their interactions with drugs [3], peptides, proteins [4], and other analytes. Liposomes are widely used as models of the bilayer core of cellular membranes. They have also been used as models for interaction studies with lipophilic substances such as doxorubicin [5] and amphotericin B [6, 7]. The structure (Figure 2) and characteristics (including surface charge, size, and lamellarity) of manufactured liposomes are easily modified by altering their phospholipid composition and the method of preparation [8]. This enables the tailoring of liposomes for specific purposes. The unique characteristics of liposomes derive mainly from the bilayer structure. Typically, phospholipids spontaneously form bilayers rather than micelles in aqueous media because of the two fatty acyl chains in their structure. In bilayers, the hydrophobic acyl chains of the phospholipid molecules face each other, while the hydrophilic head groups form the exterior of the bilayer. Their amphiphilic nature allows liposomes to be used as drug delivery systems for both hydrophilic and hydrophobic compounds.

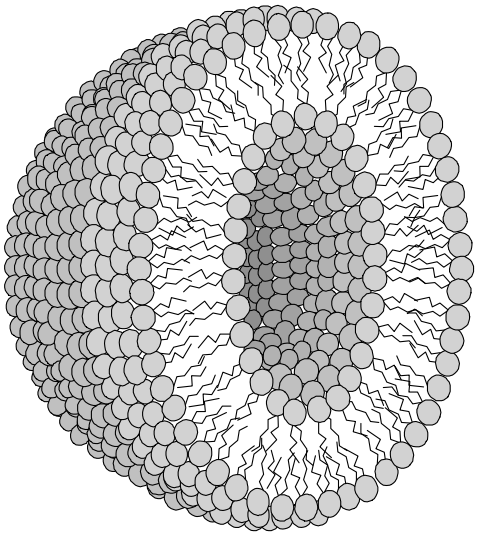


Figure 2. A through cut of a liposome showing the bilayer structure.

Liposomes can be prepared by many different methods and appear in various sizes with a variety of properties. The main methods for preparation are thin-film hydration, injection, and demulsification. In the *thin-film hydration* methods, a thin lipid film is deposited on the walls of a round-bottomed flask or test tube, an excess of aqueous solution is added, and the flask or tube is shaken. In the aqueous solution the phospholipids form multilamellar vesicles (MLV, 0.1–20 μm). Usually large MLVs are subsequently broken into smaller liposomes by mechanical treatment. Disruption of MLV suspensions with sonic energy (sonication) typically produces small unilamellar vesicles (SUV) with diameters in the range of 25–100 nm. Liposomes of defined size and homogeneity can also be prepared by application of mechanical energy (extrusion). The lipid suspension is forced several times through a filter (e.g. polycarbonate) of

defined pore size to yield small or large unilamellar vesicles (LUV) having a diameter near the pore size of the filter (100−1000 nm). In the *injection* methods the lipids, dissolved in organic solvents, are injected into a well-stirred aqueous phase. Ethanol injection is one of the most widely used injection methods and yields SUVs. In the *demulsification* methods the lipids are also introduced into an aqueous phase from an organic phase. The organic phase forms an emulsion with the aqueous phase. The lipids orient themselves in monolayers around water droplets and form liposomes when the organic phase is removed.

Bilayer-forming lipids exist in gel (solid ordered) or fluid (liquid crystalline) state depending on the relative concentration of the lipids and the temperature of the environment. When phospholipid bilayers are heated, they undergo multiple thermotropic phase transitions [9]. Much of the information on the thermotropic changes at the lipid phase transition has been obtained from X-ray diffraction and differential scanning calorimetric (DSC) studies. For an aqueous dispersion of DPPC, three transitions are observed: a sub-transition (T_s= 21.5 °C), a pre-transition (T_p= 35 °C), and a main phase transition (T_m= 41.5 °C) [9]. The most important structural change at the main phase transition temperature (T_m) is the conformational change of the acyl chain, namely, the trans→gauche rotational isomerization of methylene groups about the single C−C bonds along the acyl chains. In general, the main phase transition of a phospholipid bilayer depends on the acyl chain length, the degree of unsaturation of the acyl chains, and the backbone and headgroup structure of the phospholipid. The phase transition temperatures are also affected by external factors such as pressure, pH, hydration, and composition of the aqueous media.

2.2 Biological membranes

Phospholipids, along with cholesterol, are the major components of cellular membranes, such as human red blood cell (RBC) membranes. The most common of the phospholipid are zwitterionic PC but also SM, PS and phosphatidylethanolamine (PE) [10]. The human RBC membrane is the most studied natural membrane and is used as a model for more complex membranes. A plasma cell membrane (Figure 3) is build up from a phospholipid bilayer, in which cholesterol, proteins, and carbohydrates are embedded. Attached to the bilayer is a cytoskeleton, which holds the membrane together.

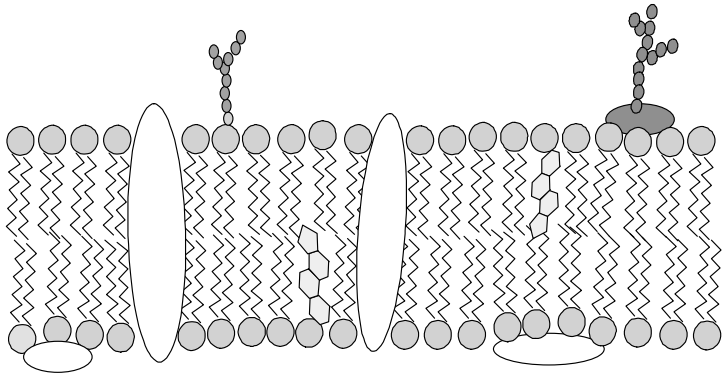


Figure 3. Schematic picture of a plasma cell membrane consisting of phospholipids, cholesterol, carbohydrates, and proteins.

The lipids in biological membranes are asymmetrically distributed between the outer and inner membrane surfaces [11]. If a biological membrane is dispersed by a detergent, symmetrical liposomes will form when the detergent is removed. Functional integral proteins are embedded in these model membranes but both the lipid distribution between the inner and outer leaflets of the bilayer and the position and orientation of the leaflet proteins will be symmetrical.

The T_m of biological membranes normally ranges between 10 and 40 °C. The transition temperature of mammalian membranes is below the body temperature, and hence these membranes are in the fluid state. Bacteria and cold-blooded animals maintain membrane fluidity by modifying the fatty acid composition through lipid synthesis and degradation. Mammals regulate the membrane fluidity by varying the cholesterol content in the bilayer. The intracellular membranes of a specific tissue in different species have very similar classes of lipids.

2.3 Cholesterol in phospholipid bilayers

As noted above, cholesterol (Figure 4) the other component of cellular membranes, is embedded in the phospholipid bilayer of the plasma cell membrane. Concentrations in the membrane range from 25 to about 50% depending on the cell type [12]. The cholesterol concentration of a human RBC membrane is 54 mol% [10]. In a study of the interactions between DPPC and cholesterol by DSC and X-ray diffraction, Ladbrooke et al. [13] showed that mixtures of DPPC and cholesterol have properties of both the gel and fluid phases of pure PC. The maximum solubility of cholesterol in PC bilayers is 66%, after which excess crystalline cholesterol separates as cholesterol monohydrate [14, 15]. Cholesterol serves as a mediator of membrane fluidity by breaking up gel domains that would otherwise make the membrane too rigid and by increasing the order of fluid membranes [16]. These properties are believed to be important in maintaining the biological activity of membranes. Furthermore, in saturated phospholipid bilayers, cholesterol broadens, and eventually eliminates, the gel→fluid phase transition, and it decreases the permeability of phospholipid bilayers in the fluid phase [17, 18].

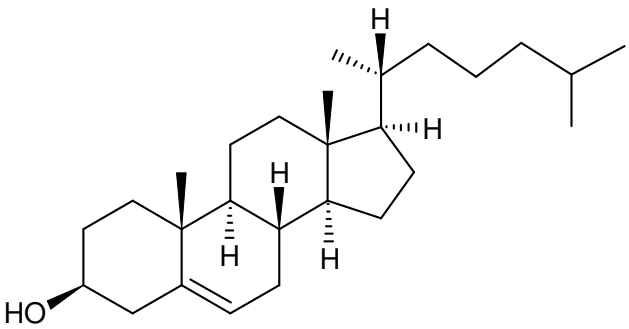


Figure 4. Structure of cholesterol.

The interaction between SM and cholesterol has been widely studied in both model membranes and biological systems [19, 20]. Cholesterol associates strongly with saturated, high-melting phospho- and sphingolipids and only weakly with highly unsaturated lipids [21]. SM has both hydrogen bond-donating and hydrogen bond-accepting functional groups, while PC has only hydrogen bond accepting groups. Because of these differences, SM is thought to have stronger intermolecular interaction with cholesterol than PC does [19, 22, 23]. Holopainen et al. [24], however, provided

evidence for lack of specific interaction between SM and cholesterol and proposed that the hydrophobic mismatch between the lipid species could explain the cosegregation of SM and cholesterol in model membranes. Membrane domains with sphingolipid- and cholesterol-based structures are called membrane rafts [20, 25, 26]. Guo et al. [27] proposed that the main reason for raft formation and the interaction of SM with cholesterol might in fact be the higher proportion of saturated fatty acid chains in SM than in other phospholipids in the natural membrane.

2.4 Divalent cations in phospholipid bilayers

Divalent cations affect the stability and structure of phospholipid bilayers and the binding and insertion of proteins [28]. The effect of calcium on negatively charged liposomes has been widely investigated. Calcium is a strong fusogenic agent, promoting the fusion of cells and liposomes [29, 30], which means that the addition of calcium ions can be used to manipulate the ability of phospholipids to form supported planar bilayers. Previously it has been shown that calcium stabilizes a POPC/PS coating on fused silica capillaries [31]. Hincha [32] investigated the aggregation of liposomes in fluorometric measurements, and found that liposomes containing 50% (w/w) of eggPC and 50% of the glycolipid digalactosyldiacylglycerol aggregated at $CaCl_2$ concentrations above 6 mM. The dispersion contained 5 mg ml^{-1} of the lipids, which is equal to ~6 mM. Membranes that contained only eggPC did not aggregate in the presence of $CaCl_2$ at concentrations up to 26 mM. Whereas the aggregated liposomes containing glycolipids were stable, aggregation destabilized liposomes containing negatively charged lipids.

Infrared spectroscopic (IR) studies by Garidel et al. [33] have shown that the binding of divalent cations (Mg^{2+}, Ca^{2+}, and Sr^{2+}) to negatively charged 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) induces a crystalline-like gel state with highly ordered and rigid all-trans acyl chains. The binding of these cations induces a tighter packing of the acyl chains, suggesting a deep penetration of the cations into the polar head group region of DMPG bilayers. The authors suggested that the primary binding site for these cations are the phosphate group of the phosphodiester moiety, but indirectly also the ester carbonyl groups are affected. The CEC results of Hautala et al. [34] indicated strong interaction between calcium and the phosphate groups in phospholipids, and they demonstrated that the phospholipid polar head group plays a significant role in phospholipid coatings on silica surfaces. In a molecular dynamics study [35], however, a sequential binding of calcium ions to the lipid carbonyl oxygens was observed. In the same simulations, a significant increase in the fatty chain order was seen. In addition, IR results have confirmed the conformational changes in the carbonyl group region of POPC and the stabilization of the gel state induced by divalent cations [36].

Very commonly, earth alkali metals such as calcium and magnesium are used to induce membrane fusion, but IR results for the interaction of zinc [37] with phospholipid membranes suggested that zinc, which is a transition metal, is an even more potent divalent cation. Metal cations have a greater influence on membranes of anionic lipids

than on neutral or zwitterionic membranes because of the stronger attractive columbic forces. It has been demonstrated that cations (e.g. Mg^{2+}, Ca^{2+}, and Ba^{2+}) elute in electrostatic ion chromatography with a *N*-dodecylphosphocholine stationary phase and water as the mobile phase in an order (Ba^{2+} <Mg^{2+} < Ca^{2+}) that differs from that of cation exchange [38]. This shows that the zwitterionic phospholipids interact with analyte ions in a unique way, based on columbic attraction and repulsion and ion-pair formation. The effect of calcium on supported bilayers has been imaged by atomic force microscopy [39]. The organization of DPPC in supported bilayers of 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) is affected by the presence of calcium. In the absence of calcium, large domains of DPPC are formed in mixtures of DPPC/DOPC and DPPC/DOPS, but in the presence of calcium, small isolated DPPC domains appear in the DPPC/DOPS mixture.

2.5 Cetyltrimethylammonium bromide in phospholipid bilayers

Surfactant molecules have various effects on the structure and properties of membranes (or membrane-like structures) [40–42]. The way a surfactant changes a phospholipid membrane depends on its structure and concentration [40, 43, 44]. Of the various possible surfactants the most commonly used are alkyltrimethylammonium salts [44, 45]. Figure 5 presents the structure of the surfactant, cetyltrimethylammonium bromide (CTAB, C16), used in this study.

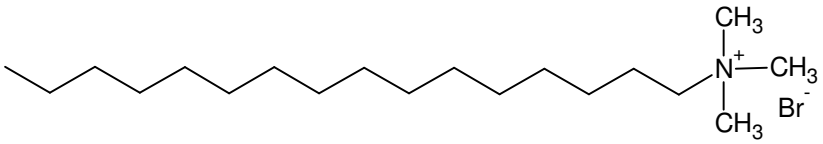


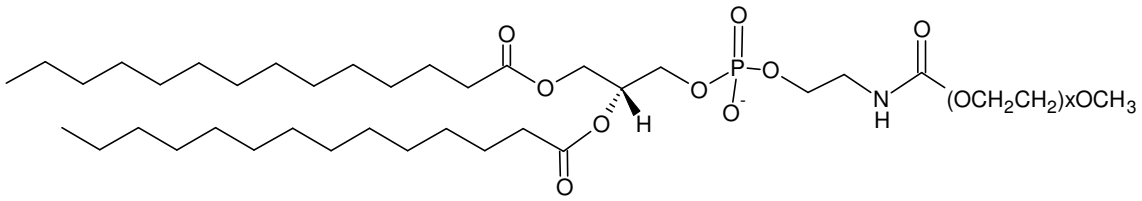
Figure 5. Molecular structure of CTAB.

In the case of zwitterionic phospholipid bilayers, alkyltrimethylammonium salts either compensate the slight negative charge of the bilayer or convey positive charge to the phospholipid structure, thereby preserving its biological activity [43]. It has been discovered that two features of the surfactant are of major importance in improving the packing of the bilayer and increasing its stability: the concentration and the length of the alkyl chain [44, 46, 47]. At concentrations well below the critical micelle concentration (CMC), alkyltrimethylammonium salts have a stabilizing effect on biological membranes and on phospholipid bilayers (in aqueous media) [44, 48, 49]. At concentrations near the CMC, the surfactant causes rapid lysis/destruction of the bilayer [44]. Both the stabilizing and destructive effects increase with the length of the alkyl chain [40, 43]. The maximum activity has been detected at about C16 [40, 43, 47]. Several investigations suggest that the reason for such behavior is the hydrophobicity of the surfactant molecule: the more hydrophobic the surfactant the greater is the entropy gain when it is incorporated from the aqueous media into the bilayer [43, 47].

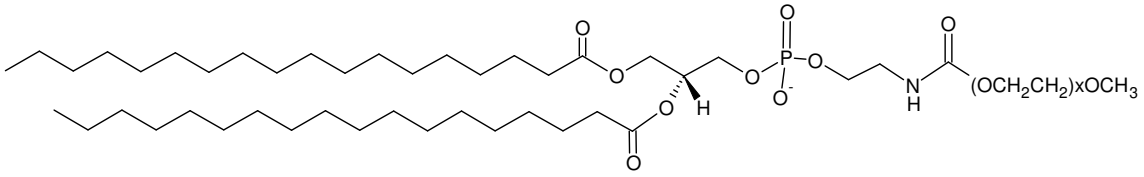
2.6 Polyethylene glycol-stabilized bilayers

Although liposomes are considered as effective drug delivery systems owing to their colloidal size, biocompatibility, large capacity for carrying drugs, and easily controllable surface and membrane properties, they are also somewhat unstable. A major breakthrough occurred in the 1990s when hydrophilic polymers were first applied to sterically stabilize the liposome outer surface. This prolonged the residence time of drug-entrapping liposomes in the blood circulation stream ensuring site-specific drug delivery [50, 51, 52].

Polyethylene glycol (PEG) is probably the most widely used stabilizer in pharmaceutical applications of liposomes because of its low toxicity, high solubility in aqueous solution, and the high flexibility of its polymer chain [53]. The protective PEG layer is thought to prevent protein adsorption to the lipid bilayer and simultaneously to act as a steric barrier for inhibiting vesicle fusion. Addition of PEG-lipids to liposomes increases the packing order in the liposomes [54, 55]. In studies on drug delivery vehicles, the focus has been on vesicles coated with PEGs of molar mass of 5000 or less. The amount of PEG in the formulations must be carefully optimized because too little PEG exhibits low protective effect in the bilayer, while too much may lead to micellization of the system. The aggregation of PEG-lipids with increasing concentration of PEG proceeds from the formation of liposomes to discoidal micelles, and finally to the formation of spherical micelles. The PEGylated lipids used in this study were sterically stabilized commercial PE-lipids with covalently attached PEG chains. This modification gives the lipid a net negative charge (dissociated phosphate group). Figure 6 presents the PEGylated lipids used in this work, namely DMPE-PEG and DSPE-PEG.



DMPE-PEG
14:0; PEG (1000, 2000, 3000)
1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)]



DSPE-PEG
18:0; PEG (1000, 2000, 3000)
1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)]

Figure 6. Molecular structures of PEGylated lipids used in this work.

3 CAPILLARY ELECTROPHORESIS

The analytical technique of interest in this work, capillary electrophoresis (CE), is a separation technique, where the analytes are separated in an electric field according to charge, mass, and structure. With automated instruments CE is easy to perform, and only small amounts of sample are required for the separation. CE has become popular because it can be used for the separation of small analytes as well as large biological molecules, such as drugs, DNA, peptides, and proteins. The instrumentation (Figure 7) consists of a capillary, two electrodes, a detector, a high voltage supply, a sample vial, and two background electrolyte (BGE) vials. The capillary that connects the vials to which the high voltage is applied is held in place by a capillary cassette. The capillary cassette is temperature-controlled by air or liquid cooling. The instrument is controlled by a computer.

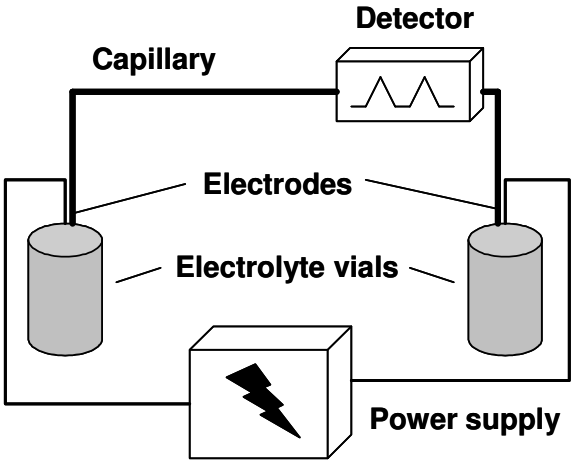


Figure 7. The CE instrumentation.

Typically, the capillary is made of fused silica and has an inner diameter of 15–250 μm and a total length of 30–100 cm. Detection is most commonly done by UV, and the field strengths are typically 200–600 Vcm^{-1}. The background electrolyte (BGE) is normally a buffer solution in water at concentration of 10–100 mM.

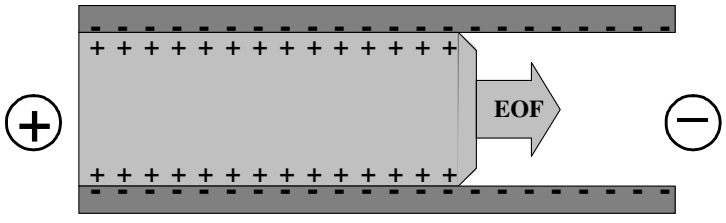


Figure 8. The electroosmotic flow in a silica capillary

When an electric field is applied, an electroosmotic flow (EOF) (Figure 8) is created in the capillary, moving the liquid towards the cathode. The EOF is created by the cations that begin to migrate toward the negative cathode, dragging the solvent along. The magnitude of the EOF varies with pH and ionic strength of the BGE and depends on the

surface charge of the inner wall of the capillary. The inner wall of the fused silica capillary consists of silanol groups, which are negatively charged at pH above 2. Under normal CZE conditions some molecules, such as positively charged proteins, adsorb onto the silica surface. To prevent this adsorption, the silanols can be shielded by coating the capillary. In the case of CEC, the capillary is coated in order to create a chromatographic phase in the capillary.

The analytes are separated according to the differences in their electrophoretic mobilities. The total mobility of an analyte, μ_{tot}, is the sum of the electroosmotic mobility in the capillary, μ_{eo}, and the electrophoretic mobility of the analyte itself, μ_{ep} (Equation 1).

$$\mu_{tot} = \mu_{ep} + \mu_{eo} \tag{1}$$

The total mobility can easily be calculated by Equation 2 once the migration time of the analyte, t_m is known. L_{tot} is the total length of the capillary, L_{det}, is the length to the detector, and U is the separation voltage.

$$\mu_{tot} = \frac{L_{tot} \cdot L_{det}}{U \cdot t_m} \tag{2}$$

The dimensionless plate number, N, calculated by Equation 3, can be considered as a measure of the separation efficiency. w_h is the peak width at half height.

$$N = 5.545\left(\frac{t_m}{w_h}\right)^2 \tag{3}$$

4 PHOSPHOLIPID-COATED CAPILLARIES

In liquid chromatography, which is a CE-related technique, liquid chromatography, liposomes were introduced to the stationary phase in a packed column as long as 30 years ago. The method is called immobilized liposome chromatography (ILC). Different approaches are available for immobilization of liposomes into the gel beads [70]. These include: steric entrapment [56-58], hydrophobic binding [59], avidin–biotin affinity binding [60-62], and covalent attachment [63]. Phospholipids can also be coupled as monolayers to the silica matrix to create an immobilized artificial membrane (IAM) [64]. When phospholipids are pumped through a reversed-phase column, the hydrophobic parts of the phospholipids are adsorbed to the hydrocarbon chains of the reversed-phase material resulting in a less permanent dynamic coating [59, 65-69].

CE studies related to phospholipids and liposomes are appearing in increasing numbers (see reviews 70–74) There are three ways in which liposomes can be used in CE: they can be studied as analytes, act as carriers, or coat the capillary. The first analysis of liposomes by CE was accomplished in 1995 by Zhang et al. [75]. For studies of liposome–analyte interactions, liposomes have been utilized as a pseudo-stationary phase [76-82]. Liposome electrokinetic chromatography (LEKC), as the method is called, allows fast and easy study of analyte–liposome interactions. However, the existence of an elution window limits the dynamic range for compounds, and the consumption of liposomes as a free-flowing pseudo-stationary phase is still high. Another drawback is that LEKC cannot be coupled directly to mass spectrometry owing to the free liposomes in the BGE. It was to overcome these drawbacks of LEKC that we were interested in using liposomes as a coating material.

Yang et al. [83] were the first in 1998, to use liposomes of PC and biotinylated phosphatidylethanolamine (PE) to coat a fused silica capillary. The phospholipids form a supported phospholipid bilayers (SPB) or a supported vesicular layer (SVL) on the silica surface (Figure 9) which can be used to separate compounds by means of electrokinetic chromatography (CEC) or to diminish interactions of the analytes with a negative silica wall. These are dynamic or semi-permanent coatings, noncovalently attached to the capillary wall, which offer advantages over permanent coatings, where the coating is covalently linked. Among the advantages are ease and speed of coating, low cost, and ease of regeneration. In 2002, Cunliffe et al. [84] used zwitterionic 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) as a semi-permanent capillary coating to prevent protein adsorption to the silica wall. Sometimes this method, where phospholipids are immobilized on a silica capillary, is called immobilized phospholipid capillary electrophoresis (IPCE). Table 2 summarizes work done on phospholipids as coating material for fused silica capillaries.

Table 2. Phospholipid coatings on fused silica capillaries. The publications are listed in order of publication year. All coatings were done on fused silica capillaries. The concentration of the liposome dispersion was < 4 mM unless otherwise mentioned.

Phospholipid coating dispersion	Electrolyte solution	Analytes	Comments	Ref.
PC/biotin-PE 98:2 mol%	Not mentioned	Acebuterol	Hydrophilic coating with avidin	83 1998
DLPC +$CaCl_2$	Tris pH 7.4	Cationic proteins	Phospholipid flush between runs	84 2002
POPC/PS 80:20 v/v%	Phosphate pH 7.4.	Neutral small drugs	Cap. first coated with agarose	85 2002
POPC/PS 80:20 mol%	HEPES pH 7.4	Neutral steroids	HEPES better than Tris or Tricine as BGE	I 2003
POPC	pH 7.5, buffer not mentioned	Charged drugs	Air dry and NaOH flush after coating	86 2003
Lipofectamine, Escort, 39mM	Tris pH 7.6	Oligonucleotides	Cationic lipids, reversed EOF	87 2003
PC/PS 80:20 mol%	Tris pH 7.4	D,L -Tryptophan	Cap. treated with M1C4, lysozyme immobilization	88 2004
PC +$CaCl_2$ or $MgCl_2$ DPPC	HEPES pH 7.4	Cationic proteins	Phospholipid flush between runs	II 2004
PC +CTAB +$CaCl_2$	HEPES pH 7.4	Cationic proteins		III 2004
POPC, 8 mM	Phosphate pH 6.2	Cationic proteins	BGE with or without POPC	89 2004
POPC/PS 80:20 mol% +$CaCl_2$	HEPES pH 7.4	Neutral steroids, phenols	Also Tris and Tricine as BGE	31 2004
PC/PS 80:20 mol%	HEPES pH 7.4	Neutral steroids	Different pH values were tested	90 2005
DOPC, ~6 mM	Tris pH 7.4	Anionic, cationic proteins	Oligomerization of DOPC	91 2005
DLPC	Ammonium pH 9.25, acetate pH 4.75		Preconcentration of proteins using a pH junction	92 2005
POPC/PS 80:20 mol%	Acetate, phos-phate, Tris pH 4.0-7.4	Neutral steroids	Small diamines were used as modifiers	93 2005
POPC/PS 80:20 mol%	HEPES pH 7.4	Anionic, neutral, cationic compounds	Also other piperazine-based buffers	94 2005
Bis-SorbPC		No CE runs	UV photopolymerization in fused silica capillaries	95 2005
DPPC/PS, Biotinyl-Cap-DPPE/PS, Biotinyl-DPPE/PS	Tris, HEPES pH 7.4	D,L -Tryptophan, D,L -PTH-serine, D,L-PTH-threonine	Avidin immobilization for chiral separtion	96 2006

Phospholipid coating dispersion	Electrolyte solution	Analytes	Comments	Ref.
PC/PS/chol PC +$CaCl_2$	HEPES pH 7.4	Neutral compounds	Effect of liposome storage time on CEC performance	97 2006
RBC ghost lipids PC/SM/chol	HEPES pH 7.4	Neutral steroids		IV 2006
POPC/fusidic acid	HEPES pH 7.4 + EDTA	Neutral steroids	Combined simulation/ experimental study	98 2006
POPC/PA, PG, PI or PS 80:20 mol% + $CaCl_2$	HEPES pH 7.4	Anionic, neutral, cationic compounds	Other polar lipid head groups than PS	34 2007
Bis-SorbPC, DOPC, DLPC, eggPC	Phosphate pH 7.4	Anionic, cationic proteins	Cross-linked, polymerized coating	99 2007
DMPC, DHPC +$CaCl_2$	MOPS pH 7.0 Tris pH 7.4	Anionic, cationic biopolymers, drugs	90 mM phospholipids Dual injection for affinity selection	100 2007
POPC/chol/fusidic acid	Hepes pH 7.4	atenolol, aldosterone, testosterone, dichlorphenamide	Interaction of fusidic acid with lipid membranes	101 2008
eggPC	HEPES 7.4 Tris 7.4	D,L-tryptophan	Immobilization of bovine serum albumin for chiral separation	102 2008
Soybean PC/chol	Tris pH 7.4	Nonsteroidal anti-inflammatory drugs	Phospholipid flush between runs	103 2008
DMPC + $CaCl_2$	Tris pH 7.4, Tris-formate pH 4.43	Cationic proteins Histone H1	Phospholipid flush between runs	104 2008
POPC/DSPE-PEG, POPC/DMPE-PEG 80:20mol%	HEPES pH 7.4	Anionic, neutral, cationic compounds FITC-AA	UV and LIF detection	V 2008

The facile and quick method for preparing the semi-permanent phospholipid coating by simply flushing the capillary with the phospholipid solution, and the versatility of the method, make CE a promising technique for studies on phospholipid–analyte interactions. With liposomes now introduced as coating material in CE techniques, characterization of the coating and the factors affecting the coating procedure are important. Liposomes and their interactions with analytes have, for example, been investigated by DSC [105, 106]. The formation of phospholipid coating on different surfaces [28, 107, 108] has been studied by AFM [108–110]. These studies have helped to clarify the progress of the coating formation and the effect of coating conditions on the final form of the coating. Several steps are involved when vesicles form a supported phospholipid bilayers (SPB) on the silica surface. [28, 36, 39, 111] First the vesicles adsorb onto the surface. Thn, if the surface coverage is high the vesicles may fuse and rupture onto the surface. The process may also include the formation of bilayer disks that fuse to form a continuous SPB (or a supported lipid bilayer, SLB, as the bilayer is also called). (See Figure 9) Viitala et al. [111] studied anionic phospholipids on silica

with a QCM and demonstrated that the formation of SVLs or SPBs is highly dependent on the lipid and solvent composition. Liposomes do not necessarily fuse and form an SPB; they can also remain intact and form an SVL.

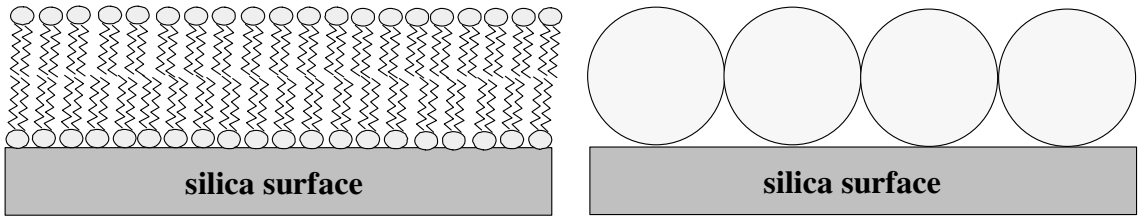


Figure 9. On the left a supported phospholipid bilayer (SPB) and on the right a supported vesicle layer (SVL).

5 EXPERIMENTAL

Details of the experimental work can be found in the original papers. Only a summary is presented here.

5.1 Chemicals

N-(2-Hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES), fluorescein isothiocyanate (FITC), POPC, bovine brain PS, eggSM, the steroids 17α-hydroxyprogesterone, androstenedione, and *d*-aldosterone, and the proteins α-chymotrypsinogen A (25 kDa, pI=9.3), lysozyme (14.3 kDa, pI=11.0), ribonuclease A (13.7 kDa, pI=7.8), and trypsin (23.3 kDa, pI=10.5) were purchased from Sigma (St Louis, MO, USA). POPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-1000] (DMPEG1000), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-1000] (DSPEG1000), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DMPEG2000), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPEG2000), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-3000] (DMPEG3000), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-3000] (DSPEG3000), DPPC, cholesterol, and eggPC (average MW 760 g mol^{-1}) were purchased from Avanti Polar Lipids (Alabaster, USA). The steroids progesterone and testosterone and the pH solutions (4.00, 7.00, and 10.00) used for calibrating the pH meter and magnesium chloride were purchased from Merck (Darmstadt, Germany). Dimethylsulfoxide (DMSO), sodium hydroxide (1.0 M), and nitric acid (1 M) were purchased from FF-Chemicals (Yli-Ii, Finland) and calcium chloride and CTAB from Fluka (Buchs, Switzerland). Zinc chloride was from Riedel-de Haën AG (Seelze, Germany), methanol from Mallinckrodt Baker (Deventer, the Netherlands), and chloroform from Rathburn Chemicals (Walkerburn, UK).

5.2 Sample and buffer preparation

The molar concentration of the BGE solution was 40 mM (ionic strength 18 mM, with pH adjusted to 7.40 with 1.0 M sodium hydroxide) of HEPES in water. Before use the BGE was filtered through 0.45 μm Millipore filters (Bedford, MA, USA) using a Millipore vacuum system. The BGE solution did not contain any liposomes.

The migration time of DMSO (0.05%, v/v) or methanol (1%, v/v) in BGE was used as a marker for the EOF. Protein stock solutions were prepared in water (2 mg ml^{-1}). The protein samples were diluted in water from stock solutions. The protein concentrations were as follows: 200 μg ml^{-1} of ribonuclease A, 250 μg ml^{-1} of lysozyme and trypsin, and 500 μg ml^{-1} of α-chymotrypsinogen A. The steroid sample was prepared from stock solutions (2 mg ml^{-1} in methanol). The steroid concentrations in the injected sample were 20 μg ml^{-1} each of aldosterone, androstenedione, and testosterone and 50 μg ml^{-1} each of 17α-hydroxyprogesterone and progesterone, all in methanol–BGE (10:90, v/v).

Amino acid stock solutions (1 mM in 10 mM $NaHCO_3$ at pH 10) were prepared. For FITC -labeling of the amino acids, 90 µL of amino acid stock solution was mixed with 10 µL of 1 mM FITC (in acetone) solution. The mixture was incubated overnight in the dark at room temperature. The final FITC-labeled amino acid concentration in the sample for injection was 0.14 µM in water. The solutions were stored in a refrigerator.

5.3 Liposome preparation

Appropriate amounts of the lipid stock solutions in chloroform were mixed to obtain the desired compositions. The mixture was evaporated to dryness under a stream of nitrogen, and traces of solvent were removed by evacuation under reduced pressure for ~16 hours. The lipid residues were hydrated in 40 mM HEPES pH 7.4 at 60 ^{o}C to yield MLVs with a lipid concentration of 0.75–4 mM, and the vesicles were maintained at this temperature for 60 min with subsequent vortexing. The resulting dispersion was processed to LUVs by extrusion 19 times through Millipore (Bedford, MA, USA) 0.1-µm pore size polycarbonate filters with a LiposoFast extruder. The liposome dispersions containing DPPC, and SM were extruded at 60 ^{o}C since the T_ms of DPPC, and SM are about 40 ^{o}C and the liposome dispersion has to be in fluid state during extrusion. The other liposome dispersions were extruded at room temperature. For the HSDSC measurements, the liposome dispersions were diluted in 40 mM HEPES (pH 7.4) to a final lipid concentration of 0.4–0.5 mM. The liposome dispersions used for coating the capillary contained 0.1–3 mM (usually 0.75 or 1.0 mM) of PC, POPC, DPPC, PS, SM, or cholesterol. For the experiments with divalent cations, 0–60 mM of $CaCl_2$, $MgCl_2$, or $ZnCl_2$ and 0–37.5 µM of CTAB were added to the POPC or DPPC dispersion after extrusion. After preparation, the liposome dispersions were stored in a refrigerator and the stock solutions of phospholipids in chloroform in a freezer.

5.4 Preparation of liposomes from red blood cell ghost lipids

Whole blood was collected by vein puncture from a single healthy volunteer with heparin used as anticoagulant. First, the RBCs were separated from heparinized whole blood by centrifugation and washed three times with a buffer consisting of 150 mM NaCl and 5 mM sodium phosphate at pH 8.0. The RBC ghosts were prepared by hypotonic shock in ice-cold lysis buffer containing 5 mM sodium phosphate at pH 8.0. After hemolysis they were washed until hemoglobin was completely removed. The ghost lipids were then extracted with a methanol/chloroform solution (2:1, v:v). The methanol/chloroform solution was evaporated to dryness under a stream of nitrogen and the ghost lipids were dissolved in chloroform at a concentration of approximately 2 mM. Liposomes (in 40 mM HEPES buffer at pH 7.4) were subsequently prepared from the ghost lipid stock solution in chloroform. The chloroform was evaporated and the ghost lipid solution was processed to LUVs by extrusion at 60 °C through 0.1-µm pore size polycarbonate filters as described above.

5.5 Instrumentation

Electrophoretic measurements were made with a Hewlett Packard ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a diode array detector (detection at 200 nm, 215 nm, and 245 nm) and air thermostating of the capillary or with a Beckman P/ACE System 2000 and 2001 equipped with a wavelength-selectable UV detector (detection at 200 nm, 215 nm, and 245 nm) and liquid thermostating of the capillary. A water bath with a circulating thermostat Lauda RE 104 (Lauda, Lauda-Königshofen, Germany) connected to the Hewlett Packard ^{3D}CE system was used to control the temperature (25 °C, 37 °C, or 45 °C) of the vial carousel.

Fused silica capillaries used for coating and in CE separations were from Composite Metal Services Ltd. (Worcestershire, UK). Dimensions were 50 μm inner diameter (ID) and 375 μm outer diameter (OD). The length of the capillary to the detector was 30.0/37.0 cm (Beckman) or 30.0/38.5 cm, 50.0/58.5 cm, or 51.5/60.0 cm (Hewlett Packard).

A nitrogen-evaporating unit (Pierce, Reacti-Therm Heating Module No 18790, Rockford, IL, USA) and a desiccator equipped with a vacuum pump (KNF Neuberger, Freiburg, Germany) were used to evaporate chloroform from the phospholipid mixture. A shaking water bath (SB-16 Techne, Duxford, UK) with a thermostat (HETO, Birkeröd, Denmark) was used to hydrate phospholipids into buffer solution, and a vortexer (Heidolph, REAX 2000, Germany) was used to accelerate hydration. Liposomes were extruded with a LiposoFast-Basic homogenizer with a pneumatic actuator or a LiposoFast low-pressure homogenizer (Avestin, Ottawa, Canada). HSDSC measurements were performed on a VP-DSC microcalorimeter (MicroCal Inc, Studio City, CA, USA). Distilled water was further purified with a Millipore Water Purification System (Millipore S.A., Molsheim, France).

5.5.1 Capillary electrophoresis

Fresh capillaries were generally flushed at a pressure of 930–940 mbar for 10 min with 0.5 M of hydrochloric or nitric acid and for 20 min with Milli-Q water. Phospholipid coating was applied to the capillary inner surface as follows: after preconditioning, the capillary was flushed for 10 min with liposome dispersion at 930–940 mbar, and then it was left to stand with the liposome dispersion for 15 min.

CE separation conditions were as follows: voltage 20 kV; temperature of the capillary cassette 25 °C, 37 °C, or 45 °C; injection of the electroosmotic marker for 2 s at 50 mbar and of the steroid and protein samples for 5 s at 50 mbar. Before runs and before each injection the capillary was flushed for 2 min with the BGE solution. A short flush with the phospholipid dispersion between runs, as in previous work involving phospholipid coating of silica capillary [84], was tested for some coatings. During a long series of runs, the quality of the BGE solution was ensured by change of the buffer vials after five to ten run.

The stability of the coatings was measured by performing successive runs with the EOF marker in freshly coated capillaries. The stability of the coatings was measured indirectly by monitoring the change in EOF during successive runs.

The zeta potentials and particle sizes of PEGylated and nonPEGylated POPC aggregates were determined with a Zetasizer 3000 instrument (Malvern Instruments). The samples were diluted in HEPES buffer solution (concentration 20 mM at pH 7.4) until the final concentrations were 0.25 mM (sample volume 2 ml). Measurement conditions and parameters were as follows: 25°C, dielectric constant 79.7, and viscosity 0.89 mPa·s. Electrophoretic mobilities were converted into zeta potentials with use of the Smoluchowski equation.

5.5.2 Differential scanning calorimetry

HSDSC measurements were performed on a VP-DSC microcalorimeter at an external pressure of ca. 180 kPa. The cell volume was 0.507 ml. The instrument response time was set at 5.6 s. Scans were performed from 10 to 60 °C at a heating rate of 60 °C h^{-1}. Before each scan, the sample was kept at 10 °C for 30 min. The lipid suspensions were degassed under vacuum before being loaded into the VP-DSC microcalorimeter. Data were corrected for instrument response time and analyzed with the software supplied by the manufacturer.

5.5.3 Asymmetrical flow field flow fractionation

The AsFlFFF channel was constructed in-house in a manner similar to that of other groups [112-115]. A regenerated cellulose acetate ultrafiltration membrane with a molar mass cut-off of 10 kDa (DSS-RC70PP, Nakskov, Denmark) was laid on top of the porous frit. A 500-μm-thick Mylar™ spacer, with the channel shape cut out, was placed between the ultrafiltration membrane and the upper glass plate. The nominal channel dimensions were 38 cm × 2 cm × 500 μm. An HPLC pump was used to move the carrier liquid, and samples (5.0%, v/v) were introduced to the channel at 1.0 ml min^{-1} for 5 min by another HPLC pump. During the injection–relaxation–focusing period, carrier liquid was delivered from the front and backside of the channel at 3.3 ml min^{-1} for 12 min. The outlet flow from the channel was monitored with a UV/Vis detector (HP1050 model 79853C, Tokyo, Japan) at 254 nm. Unless otherwise specified, the flow rates at the main and cross-flow outlets were 2.25 and 0.75 ml min^{-1}, respectively.

5.5.4 Dynamic light scattering

Size distributions of the lipid aggregates were measured by dynamic light scattering (DLS) with a Brookhaven Instruments BI200SM goniometer and a BI-9000AT digital correlator (Brookhaven Instruments, Holtsville, NY, USA). The extruded samples were diluted in HEPES buffer solution (concentration 20 mM at pH 7.4) to a final concentration of 0.5 mM. The DLS measurements were made at 20°C, the viscosity of the sample solution was approximately 1 mPa·s, and the refractive index was 1.33. The

laser light (Argon Lexel Laser of wavelength 488 nm) was detected at 90°. Three replicate measurements were made and the mean average particle diameter was obtained with the CONTIN software.

Particle sizing was also detected at 90° with a He-Ne laser at 633 nm. Three replicate measurements of ten runs (in total 30 runs) were made on each lipid dispersion, and mean average particle diameters were determined with the use of the CONTIN algorithm. The sizes presented are based on the volume distributions.

5.5.5 Electron microscopy

For study of the packing of PEGylated lipid aggregates on silica, portions (3 μL) of extruded POPC/DSPEG1000, POPC/DSPEG2000, and POPC/DSPEG3000 80:20 mol% samples at 1 mM in 20 mM HEPES pH 7.4 were pipetted onto a holey carbon film on 400-mesh copper support grids (Quantifoil R2/2). Samples were blotted with Whatman no. 41 filter paper and then vitrified by plunging into liquid ethane in a home-made guillotine [116]. The vitrified samples were transferred to a Gatan 626 cryoholder and maintained at -180 ºC during imaging in a Tecnai F20 field emission gun transmission electron microscope (Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki) at 200 keV. Micrographs were recorded on Kodak SO163 film under low dose conditions at a magnification of 50000 x.

5.5.6 Atomic force microscopy

The interaction between PEGylated lipid aggregates and the outer surface of fused silica capillaries (the polyimide layer had been removed) was studied by AFM using an Autoprobe CP Research AFM (ThermoMicroscopes, Sunnyvale, CA, USA), operated in intermittent-contact (tapping) mode. All AFM measurements were conducted at ambient temperature and humidity using MikroMasch NSC21 triangular Si cantilevers ($k = 1$ N m^{-1}), with typical end radii <10 nm and cone angles <30° for the probe tips. Capillaries were prepared for each separate lipid dispersion by flushing for 5 min with 0.5 M nitric acid and then for 10 min with water. After this the capillaries were left to stand in lipid aggregate dispersion for 15 min. Measurements on the outer walls of the capillaries were carried out directly after capillaries were removed from dispersions.

5.5.7 Measurements with a quartz crystal microbalance

A KSV multi-frequency QCM-Z500 (KSV Instruments, Helsinki, Finland) was used to monitor adsorption of the PEGylated lipid aggregates on a Si-coated quartz crystal. Measurement with the QCM-Z500 instrument is based on impedance analysis. The contacting adsorbed layer and bulk liquid phase create mechanical perturbations in the quartz crystal and alter its electrical characteristics. Thus, by measuring the impedance over the crystal and using equivalent circuit analysis, one can relate the electrical impedance of the quartz crystal to the mechanical properties of the contacting adsorbed layer and liquid phase. If the layer on the crystal surface is rigid, coupled strongly to the

crystal movement, and homogeneously deposited over the crystal surface, the decrease in frequency is proportional to the mass of the adsorbed layer, as shown by Sauerbrey [117] in 1959.

If the adsorbed layer does not fulfill the above requirements because the added mass is soft and/or not properly attached to the underlying surface, and therefore it is not fully coupled with the crystal oscillation, there is a deviation from the Sauerbrey equation. In such cases, measuring the electrical properties of the quartz crystal and the adsorbed layers at multiple overtones is a useful way to determine the mechanical properties of the layer. By using equivalent circuit modeling, one can then estimate the mechanical properties (mass, density, thickness, viscoelasticity) of the added layers on the quartz crystal. [111, 118] The modeling in this study was performed using proprietary QCM Impedance Analysis software (KSV Instruments, software version 2.30). Data was simultaneously acquired at the fundamental frequency of 5 MHz (N = 1) and at several overtone frequencies (15, 25, 35, 45, and 55 MHz). If not stated otherwise, the changes in the measured data of the third overtone (N=3; f=15 MHz) are reported.

The silica-coated quartz crystals used in the QCM measurements were prepared by evaporating onto the gold-electrode-coated 5 MHz quartz crystal a 50-nm thick layer of Si, which spontaneously oxidizes to SiO_x upon air exposure. Before measurements the silica-coated quartz crystal was immersed in 0.5 M nitric acid for about 15 minutes and flushed with pure water. Immediately after this the crystal was placed in the QCM measuring chamber and the chamber was filled with pure water and flushed 3-4 times with pure water until the frequency signal was stable. Phospholipid coating was then applied to the chamber by flushing it with the vesicle dispersion.

6 RESULTS AND DISCUSSION

The main purpose of this study was to develop a method for coating phospholipids onto CE capillaries. Stabilization of the coating was pursued through the addition of divalent cations, CTAB, and PEGylated lipids. As well, the effect of temperature on the coating stability was investigated. DSC experiments were carried out to characterize the thermotropic behavior of the liposomes. In an attempt to move toward more natural membranes, the effect of cholesterol was studied and the capillary was coated with human RBC ghost lipids. Emphasis was on the rigidity and stability of the phospholipid coating. In the following, first the simple method developed for coating a fused silica capillary with phospholipids is described, and then how the addition of divalent cations and CTAB affects the rigidity of the phospholipid membrane and the stability of the coating for CE. The effect of PEGylated lipids on phospholipid bilayers as studied by CE, AsFlFFF, and spectroscopic and microscopic techniques is reported. Finally, the successful coating with RBC ghost lipids, and the interaction of steroids, proteins, and small molar mass drugs with the phospholipid bilayers are described.

6.1 Development of the coating procedure

The coating procedure was developed through an investigation of different methods of preconditioning the capillary and of different coating times and phospholipid concentrations. Related to this, experiments were carried out on the best way to store capillaries.

6.1.1 Preconditioning of the capillary

Different methods of preconditioning the fused silica capillary were tested: flushing with sodium hydroxide, flushing with sodium hydroxide and acid (hydrochloride or nitric acid) and flushing only with acid. Since flushing with acid gave slightly better coating results (data not shown), an acid flush with hydrochloric or nitric acid was chosen. After the preconditioning with acid (and water), the capillary was flushed with BGE solution for five minutes. There was no clear improvement in the coating, however, and this preconditioning step was excluded.

6.1.2 Optimization of the phospholipid coating method

The coating method was optimized for the coating time, including the flushing with the liposome coating solution and the waiting time, and for phospholipid concentration.

6.1.2.1 Coating time

When the capillary was flushed with the coating solution for 5, 10, or 20 min [I], there was no significant difference in the mean electroosmotic mobilities (the relative standard deviation, RSD, was < 7% overall). Accordingly, 10 min was selected as long

enough to flush the capillary thoroughly but short enough to keep the method rapid and not waste the liposome dispersion.

The formation of bilayers on surfaces during coating processes has been observed for double-chained surfactants [119] and lipids with two alkyl chains [84, 109]. However, the formation of the bilayer often takes some time [110]. The effect of waiting 5 min to 23 h before removing the liposome dispersion from the capillary was studied for anionic POPC/PS coatings [I]. Measurement of the EOF after successive injections of methanol suggested that there was no difference in the coating stability after 5 and 15 minutes waiting time. The difference in the mean electroosmotic mobilities was less than 1.5%. The corresponding RSD values as calculated for 36 runs were 2.8 and 1.5%, respectively. When the capillary was kept filled with the liposome dispersion for 23 h, the capillary became slightly unstable, as indicated by a fluctuation of the EOF (RSD of 4.5%). Thus, 15 minutes waiting time was considered sufficient to achieve a stable coating. This was also in agreement with previous studies [110].

The effect of waiting time (15 min, 2 h, 15 h, and 65 h) was also tested for eggPC/CTAB/Ca^{2+} coatings [III]. These results similarly indicated that a sufficiently stable coating is achieved if the capillary is kept filled with the coating solution for just 15 min.

The following method was chosen for the subsequent CEC studies: 10 min 0.5 M acid flush + 15 min water flush + 10 min liposome dispersion flush + 15 min waiting time with the liposome dispersion in the capillary. As mentioned, the stability of the coating did not improve markedly with prolongation of the coating time. However, the waiting time did have an effect on the interactions between the phospholipid membrane and model proteins, and a total coating time of 15 h was concluded to be best for the protein separations on capillaries coated with eggPC/CTAB/Ca^{2+} [III].

In a recent study of the effect of ionic strength and liposome preparation on phospholipid coating formation, Gulcev and Lucy [104] showed that the coating of the capillary was faster when Tris buffers of higher ionic strengths (2.5 $\rightarrow$ 40 mM) were used. Possibly, if the ionic strength of the HEPES buffer used in this study had been higher, the coating process would have been faster. Nevertheless, 15-min coating time can be considered short as compared with the time required to coat capillaries covalently.

6.1.2.2 Phospholipid concentration

As a further step in the optimization the phospholipid concentration in the coating step was varied. The tested phospholipid dispersion contained PC and metal ion in molar ratio 1:20. Dispersions containing 3 mM PC resulted in stable coatings [I]. Phospholipids are of relatively high cost, however, and it was desirable to find the lowest concentration that would give a good coating in the capillary [II]. The estimation of coating stability was based on the run-to-run electrophoretic mobility and the plate number of the lysozyme used as model basic protein. Figure 10 (Figure 1 in paper II)

shows that there was no phospholipid coating in the capillary at PC concentrations below 0.2 mM. The best results (highest plate numbers) were obtained with 0.75 mM PC. The EOF was fairly stable at all PC concentrations from 0.25 mM PC to 3 mM PC (Figure 10a), showing that the net charge of the surface was almost constant, and the silanols on the fused silica capillary were shielded by the PC coating. Fluctuations in the plate number of lysozyme (Figure 10b) were probably due to lysozyme interacting in different ways with the PC coating. The thickness of the coating may have varied with the PC concentration, or lysozyme may have interacted with the calcium added to the PC dispersion, or with PC phospholipids leaking out from the capillary.

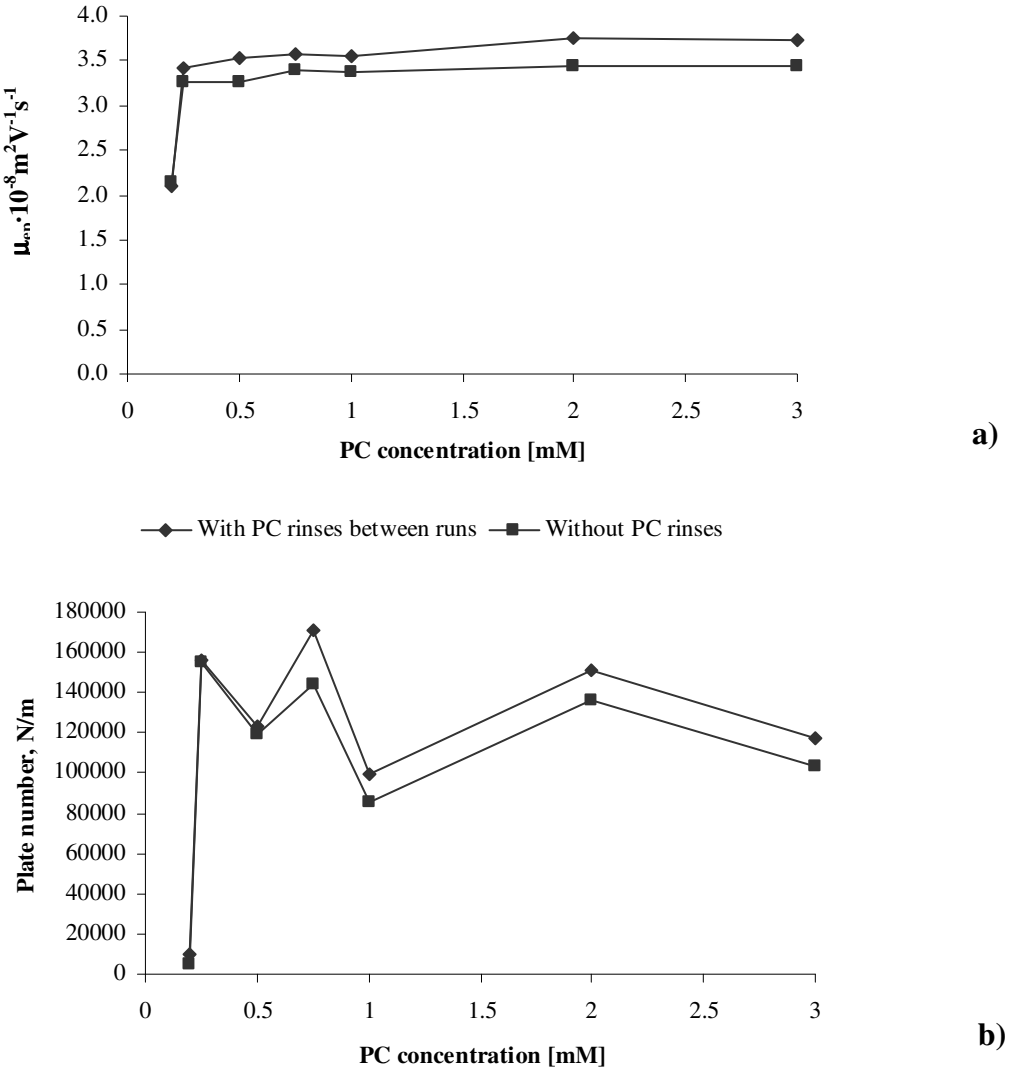


Figure 10. Effect of PC concentration (0.1–3 mM) on the coating. The PC/Ca^{2+} ratio was 1:20. Capillary 30.0/38.5 cm, ID/OD 50/375 μm. Injection of 250 μg ml^{-1} lysozyme in water for 5 s at 50 mbar. a) The electrophoretic mobilities and b) the plate number for lysozyme. Separation conditions: 20 kV, 25 °C, UV at 215 nm.

When the effect of phospholipid concentration was investigated with DMPC prepared as SUVs, Gulcev and Lucy [104] found that even concentrations of 0.1 DMPC were sufficient to coat the capillary. Interestingly, they found that when DMPC were used as

LUV or MLVs the coating process was slower and the EOF was not suppressed as much as when SUVs where used. The results are not fully comparable with those of our study, however, since we used LUVs made of POPC with unsaturated acyl chains and they used DMPC (T_m 24 °C) with saturated short acyl chains. Their runs were made at 25 °C, i.e., just above the transition temperature. We also studied the saturated DPPC (T_m 41.5 °C) (see section 6.2.5) LUVs, but below the phase transition temperature, and obtained even lower EOFs for this rigid gel phase coating than Gulcev and Lucy.

6.1.3 Conditions for capillary storage

Since reuse of capillaries is desirable, tests were made to determine if the POPC/PS coating could withstand overnight storage without losing its stability [I]. When the capillary was stored in water, the coating was apparently dissolved, since the EOF measured the following day was almost as fast as with an uncoated capillary. When the capillary was stored in the liposome dispersion, the coating evidently remained attached to the capillary wall, but the EOF fluctuated strongly. When the capillary was kept in the HEPES BGE solution for about 13 h, no significant loss of coating occurred as evidenced by the more or less stable EOF. The capillary was also stored in HEPES buffer solution for about 60 h, and even after such a long time the coating was stable and there was only a slight increase in the EOF (2%). Nevertheless, a freshly coated capillary should be employed for each series of runs to ensure reliable data.

Tests on the stability of the coating were also done with POPC/PEG aggregates. The coated capillary was kept in the BGE solution for one day, one week, or two months. The EOF values did not change much after storage for one day (~$1.0 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$, RSD 2.4%, n=9; the EOF of an uncoated capillary under the same running conditions is approximately $5 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$) and were slightly higher (~$1.4 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$) but still repeatable (RSD of EOF 1.0% with n=9) after one week. After storage for two months in the BGE solution, the PEGylated lipid coating had changed and was partly dissolved; the EOF values were now about $1.9 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$ (RSD 3.2%, n=9), but clearly some was still present in the capillary.

6.2 Stabilization of the phospholipid coating

A major part of the work involved determining and improving the stability of the coating. When the stability of the PC coating was confirmed to be unsatisfactory, attempts were made to improve the stability through adding metal cations and CTAB, working in the gel phase, adding cholesterol and adding PEGylated lipids.

6.2.1 Stability of phosphatidylcholine coatings

The stability and charge of the coating in a phospholipid-coated fused silica capillary was determined by measuring the repeatability of the EOF. When the silica surface, which is originally negatively charged, is coated with liposomes, the liposomes shield the negative silanol charges and the EOF is suppressed. In a pure DPPC-coated capillary, [IV] the EOF was suppressed from about 5 $\times 10^{-8}$ $m^2V^{-1}s^{-1}$ in the uncoated capillary to as low as 0.2–1 $\times 10^{-8}$ $m^2V^{-1}s^{-1}$. Figure 11 (Figure 3 in paper I) shows the EOF values obtained for a large number of runs in an uncoated capillary, a capillary coated with 80:20 mol% POPC/PS, and a capillary coated with 100 mol% POPC.

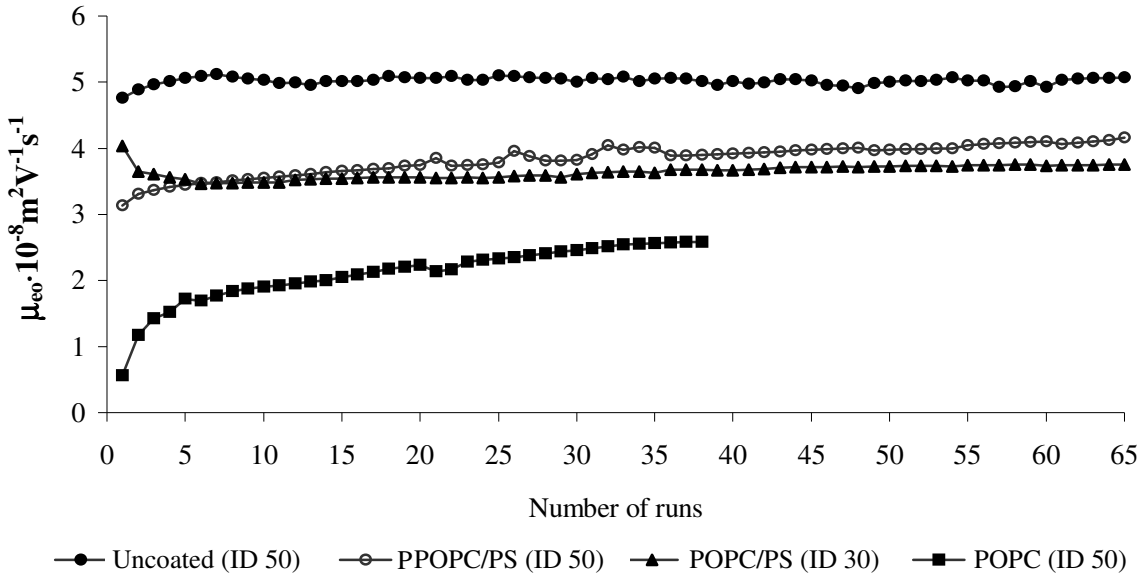


Figure 11. Repeatability of the EOF, μ_{eo}, in uncoated and in 1 mM POPC- and 80:20 mol% POPC/PS-coated capillaries (51.5/60.0 cm) using 40 mM HEPES at pH 7.4 as liposome solvent and BGE solution. Injection of methanol for 5 s at 50 mbar. Separation conditions: voltage 20 kV, temperature of capillary cassette 25 °C, detection 200 nm.

A 2-min flush with BGE solution was made before each injection, and the duration of each run was 12 min except for 100 mol% POPC. In the case of POPC the running times were from 50 min for runs 1–8 and 30 min for runs 9–38. In the case of the 80:20 mol% POPC/PS coating there are two curves: one for a capillary with 30 μm ID and one for a capillary with 50 μm ID. The EOF in the 80:20 mol% POPC/PS-coated capillaries was almost as stable as the EOF in the uncoated capillary. The EOF in the POPC/PS-coated capillaries increased slightly as the capillary was used, probably because some liposomes leaked out from the capillary or the nature of the coating changed. Although the EOF in the 100 mol% POPC-coated capillary was suppressed at the outset, it increased after a few runs. Presumably, the adsorption of the liposomes onto the silica surface was not strong enough to withstand the applied electric field or the buffer flushing between the runs. Under these conditions, POPC liposomes alone cannot provide a stable phospholipid coating, but 80:20 mol% POPC/PS liposomes seemed to work very well.

The mean EOF values for a large number of runs and for the different phospholipid coatings are summarized in Table 2. The repeatability was good for the 30-μm ID 80:20 mol% POPC/PS-coated capillary, as can be seen from the small RSD value for the very long series of runs. The RSD for the 100 mol% POPC capillary was poor (20.7%). The large RSD value was not the result of a constant fluctuation of EOF but rather to a systematic increase in EOF (see Figure 11). EOF values also fluctuated in the uncoated capillary, and slowly increased as the capillary was used. With a 51.5/60.0 cm uncoated capillary (ID 50 μm), 65 injections of methanol gave 1.94% RSD for the EOF.

Table 2. EOF (μ_{eo}) in capillaries coated with different 1 mM liposome dispersions.

Capillary	**Average $\mu_{eo} \cdot 10^{-8}$/ $m^2s^{-1}V^{-1}$**	**RSD (%)**	**# of runs**
Uncoated	5.02	1.9	65
POPC 100 mol%	2.06	20.7	35
POPC/PS 80:20 mol% (ID 30 μm)	3.64	2.8	65
POPC/PS 80:20 mol% (ID 50 μm)	3.83	6.0	65

The stability of a more heterogeneous eggPC coating was also studied [III]. Coating the capillary with eggPC caused the EOF to decrease to about one-fourth the value for the uncoated fused silica capillary (from $3.6 \cdot 10^{-4}$ to $0.9 \cdot 10^{-4}$ $cm^2V^{-1}s^{-1}$), which is similar to the results obtained with the POPC coatings [I]. The EOF was still toward the cathode, i.e., the capillary wall was still negatively charged. This result is in agreement with literature data, and suggests that the zwitterionic bilayers on the fused silica capillary wall have a slightly negative charge [120, 121]. The negative charge is due either to adsorption of negative ions from the solution or to non-Columbic electrostatic potential deriving from strong hydration of the phospholipid bilayer [78, 120].

The interactions between PC liposomes and the capillary wall were strong enough to yield a semipermanent coating; that is, liposomes were not present in the BGE solution. Still, the stability of the POPC and eggPC coatings was not very good. Even though the capillary was flushed with liposome dispersion between the runs (1 min with liposome dispersion, followed by 1 min BGE flush), the EOF increased slowly but steadily during six consecutive runs (90 min of high voltage (HV) exposure). The change was more than 5%. Evidently, the coating was slowly leaking out of the capillary.

The separation of steroids (aldosterone, androstenedione, testosterone, 17α-hydroxyprogesterone, and progesterone) was successful when liposomes containing POPC alone or POPC and PS were used as coating. The steroids were completely separated when the capillary was coated with POPC, but the coating was not stable and the migration times were long. Even though the EOF fluctuated, the electrophoretic mobilities of the analytes were almost constant in successive separations with all coatings. Figure 12 shows an electropherogram of the separation of the five steroids for the 18th run on the POPC-coated capillary.

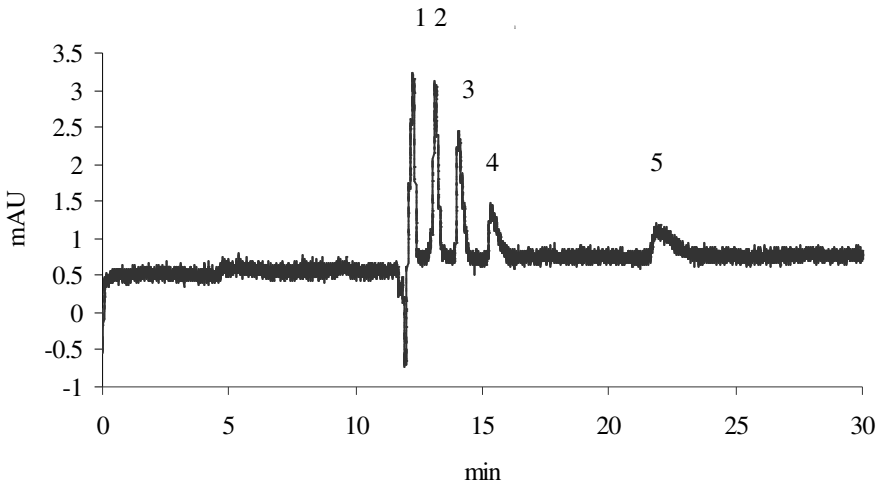


Figure 12. Separation of five steroids (1 aldosterone, 2 androstenedione, 3 testosterone, 4 17α-hydroxyprogesterone, and 5 progesterone in 10:90% (v/v) methanol/BGE) on a POPC-coated capillary (51.5/60 cm). Injection for 5 s at 50 mbar. Separation conditions: 20 kV, 25 ºC, 245 nm, BGE 40 mM HEPES at pH 7.4.

Baseline separation of the steroids was achieved when the capillary was coated with 100 mol% POPC or 80:20 mol% POPC/PS. Although the POPC/PS coatings were reasonably stable, our interest was neutral phospholipid coatings. Since PC alone did not yield a stable coating, the next logical step was to investigate how the coating stability could be improved. One way of improving the coating stability of neutral phospholipid membranes is to increase the rigidity of the membrane through the addition of divalent cations, CTAB, cholesterol or PEGylated lipids, or by working with phospholipids in the gel phase. These approaches were tested and the results are reported below.

6.2.2 Addition of divalent cations

Since calcium acts as a strong fusogenic agent for bilayer formation and performed well in earlier phospholipid coating studies [39, 84, 109, 122], study was made of the effect on stability of adding calcium [II, III] or magnesium [II] to the liposome dispersion during the coating step. The PC concentration was held constant at 0.75 mM and metal ions were added up to a molar ratio of 1:20 for PC/metal ion. The EOF as a function of number of injections is shown in Figure 13. In the first 10 runs, the capillary was flushed for 1 min with the liposome dispersion and 1 min with the BGE solution before each injection. In the following 10 runs, it was only flushed with the BGE solution, for 2 min before each injection. The dotted line (Figure 13) indicates the change in flushing conditions.

Both calcium and magnesium affected the phospholipid coating. For PC/Ca^{2+}, the EOF was most strongly suppressed at a molar ratio of 1:3 and the stability of the coating (smallest RSDs for the EOF values) was then the best. In the case of PC/Mg^{2+}, molar ratios of 1:3 and 1:10 gave the best and equally good results. Increasing the metal

concentrations to a molar ratio of 1:20 did not improve the coating stability, evidently because the PC membrane was saturated with metal ions.

Calcium and magnesium form complexes with the phosphate groups of phospholipids and dehydrate them [34, 36], or they are immobilized into the membrane and cause conformational changes in the carbonyl region of PC [35]. A combined effect is also possible. The molar masses of calcium and magnesium are 40.1 and 24.1 g mol^{-1} and the ionic radii are 99 and 72 10^{-2} nm, respectively. In the case of immobilization in the phospholipid membrane, the size of the metal ion might determine how well it can be immobilized. Since magnesium is smaller it can more easily penetrate into the membrane, and a higher concentration of magnesium than of calcium can stabilize the coating.

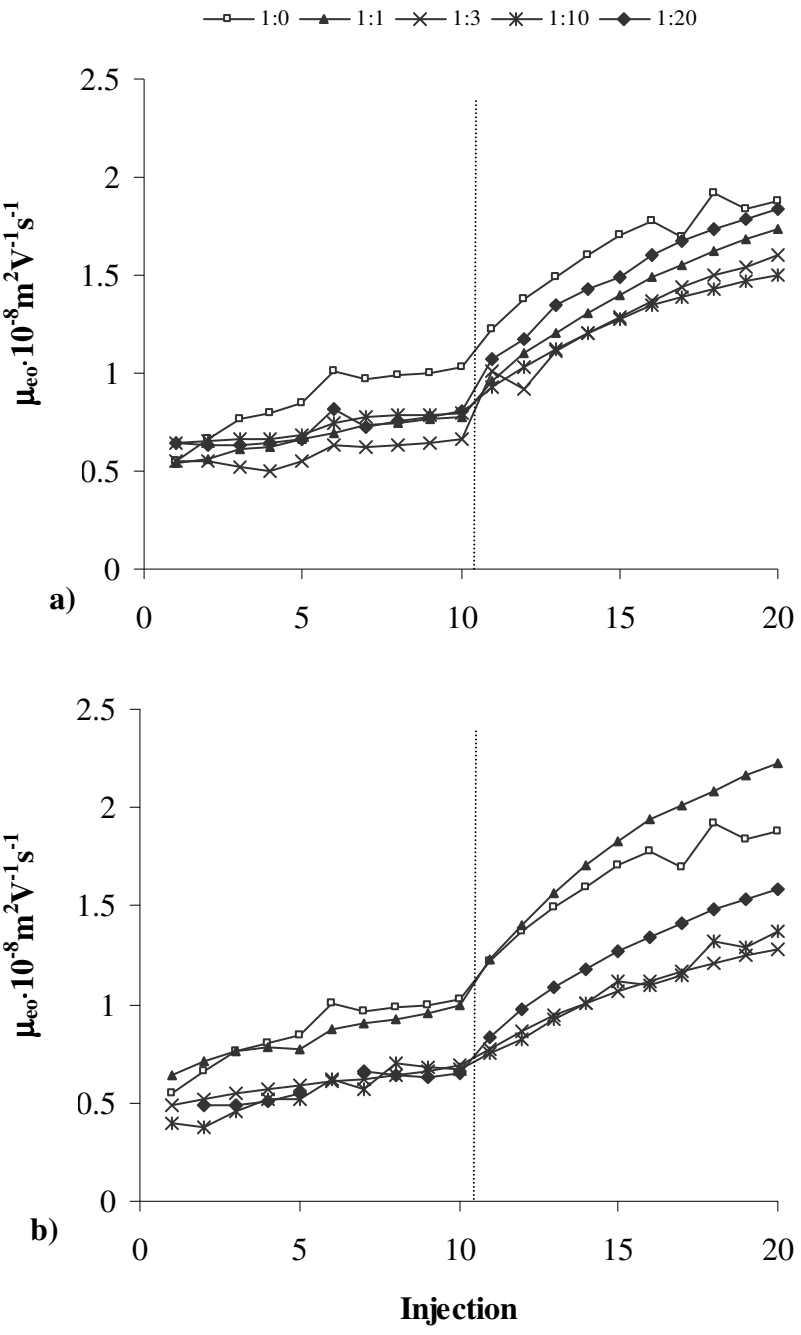


Figure 13. Effect of a) Ca^{2+} and b) Mg^{2+} on the coating stability. The concentration of PC was 0.75 mM and the PC/metal ion molar ratio varied between 1:0 and 1:20. Capillary 30/38.5 cm, ID/OD 50/375 µm. Injection of 0.05% DMSO in BGE solution for 2 s at 50 mbar. Separation conditions: 20 kV, 25 °C, 215 nm. The dotted line indicates the change in flushing conditions from flushing 1 min with liposome solution plus 1 min with BGE solution to flushing 2 min with BGE solution.

As seen from the EOF values in Figure 13 (Figure 2 in paper II), neither metal ion was able to stabilize the coating well enough without a liposome dispersion flush between the runs. This was as expected, since the electrostatic interactions of divalent cations with zwitterionic liposomes such as PC [37] are weaker than interactions with anionic liposomes [31]. As noted by Garidel et al. [33], the addition of calcium or magnesium to lipid bilayers makes the bilayers more gel-like. Evidently, interaction with multivalent cations affects the packing of phospholipids in the bilayer [123].

It is difficult to study basic proteins by electromigration techniques at neutral pH on an uncoated fused silica capillary because of the strong interactions between the negative silanol groups and the positively charged proteins. The shielding of silanols was examined by coating the capillary with eggPC [III], PC/Ca^{2+}, PC/Mg^{2+} [II], PC/CTAB, and PC/CTAB/Ca^{2+} [III]. The interactions between the phospholipid coating and basic proteins were studied.

A sample containing lysozyme, trypsin, ribonuclease A, and α-chymotrypsinogen A was injected into a PC/Ca^{2+}-coated capillary (see Experimental section for molar masses and pI values). Figure 14 shows the plate numbers for the four proteins as a function of PC/Ca^{2+} molar ratio for separations with liposome flushes before each injection. The plate numbers are the average of six protein injections.

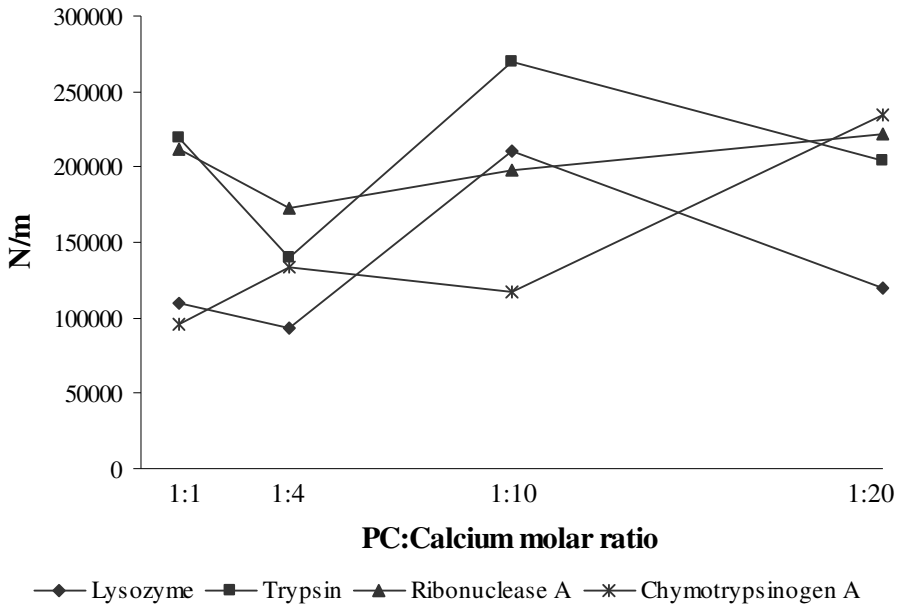


Figure 14. Plate numbers for lysozyme, trypsin, ribonuclease A, and α-chymotrypsinogen A as a function of PC/Ca^{2+} ratio. The capillary was flushed for 1 min with liposome dispersion and for 1 min with BGE before each injection of proteins for 5 s at 50 mbar. The plate numbers are an average of six injections. BGE: 40 mM HEPES at pH 7.4. Separation conditions: capillary 30/37 cm, ID/OD 50/375 µm; 20 kV; 25 °C; 215 nm.

As can be seen from Figure 14 (Figure 4 in paper II), the plate numbers for ribonuclease A and α-chymotrypsinogen A increase with the amount of calcium. This might be because the coating becomes more positively charged at higher PC/Ca^{2+} molar ratios

and repels the positively charged proteins. For lysozyme and trypsin the plate numbers reach a maximum at a molar ratio of 1:10. Lysozyme and trypsin interacted to some extent with the phospholipids or calcium, or with both.

Most proteins contain some histidine or tryptophan on their surface and form complexes with heavy metals [124]. In immobilized metal ion affinity chromatography (IMAC), proteins are eluted from the gel in an order depending on the histidine or tryptophan content on the surface of the protein. Any metal ion with an excess of d-electrons is suitable for IMAC. The most popular metals are Ni^{2+}, Cu^{2+}, Zn^{2+}, and Co^{2+}, but Ca^{2+}, which is an earth alkali metal, has been used as well. Since proteins have an affinity to calcium, the decrease in plate numbers for lysozyme and trypsin at PC/Ca^{2+} molar ratio 1:20 might be due to the excess calcium in the coating. The RSDs for the migration times of the proteins in PC/Ca^{2+}-coated capillaries were less than 4.5% for the runs with liposome flush before each injection, and as much as 9% when the capillary was only flushed with BGE before each injection.

For further study of the phospholipid coating, comparison was made of capillaries coated with PC/Ca^{2+} (1:20) and PC/Mg^{2+} (1:20) (Table 3, Table 1 in paper II)). For both calcium and magnesium, the runs with liposome flush before each injection gave better RSDs of the migration times. As can be seen, in general the RSDs for magnesium are twice as large as the RSDs for calcium.

Table 3. The RSD of the migration times for proteins separated on capillaries (30/37 cm, ID/OD 50/375 μm) coated with PC/Ca^{2+} or PC/Mg^{2+} in molar ratio 1:20. Six runs were made with liposome flushes between the runs, and six runs with BGE flush between the runs. BGE solution: 40 mM HEPES at pH 7.4. Separation conditions: 20 kV, 25 °C, detection 215 nm.

	PC/Ca^{2+}, 1:20		**PC/Mg^{2+}, 1:20**	
	Liposome flush	BGE flush	Liposome flush	BGE flush
Lysozyme	0.13 %	1.08 %	0.3 %	2.07 %
Trypsin	0.43 %	1.77 %	0.3 %	2.59 %
Ribonuclease A	0.28 %	2.48 %	0.5 %	3.84 %
α-Chymotrypsinogen A	0.59 %	1.99 %	1.3 %	4.02 %

Figure 15 (Figure 5a in paper II) shows the separations of proteins on PC/Ca^{2+}(1:20)-coated capillary. Lysozyme is tailing, probably because of the above-noted interactions with the coating. Proteins adsorb onto phospholipid surfaces [125], but to a lesser extent on phospholipids like zwitterionic PC. Holmlin et al. [126] showed that zwitterionic self-assembly monolayers terminating in a phosphatidylcholine headgroup reduce the adsorption of proteins from aqueous buffer but cannot eliminate it. α-Chymotrypsinogen A decomposes slightly under the influence of trypsin, which works as a proteolytic enzyme. The other proteins in the sample may also be digested by trypsin to some degree. The presence of trypsin did not cause any major problems, however, since the sample was only used at 25 °C for a few hours, and a fresh sample was prepared each time a new coating was tested.

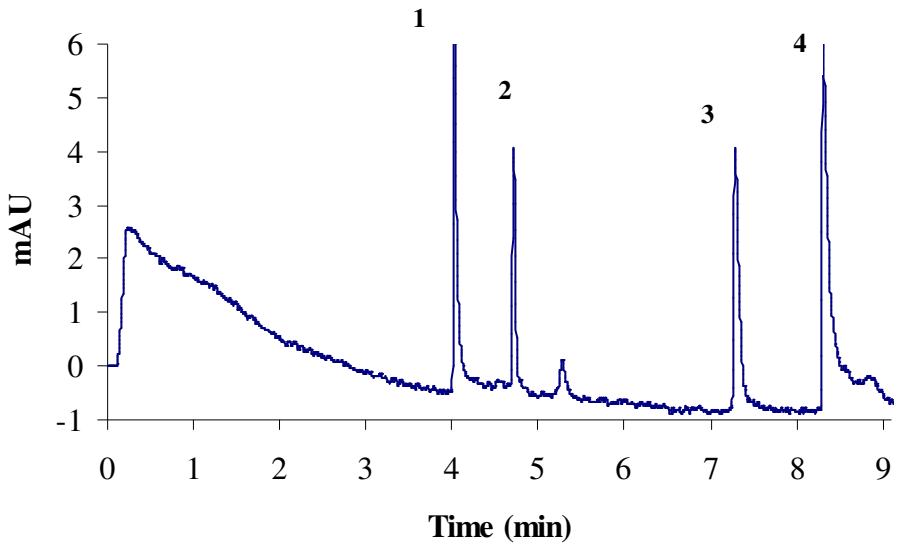


Figure 15. Protein separations on a fused silica capillary (30/37 cm, ID/OD 50/375 µm) coated with a) PC/Ca^{2+} (1:20) and b) PC/Mg^{2+} (1:20) in 40 mM HEPES at pH 7.4. Injection of 1 lysozyme, 2 trypsin, 3 ribonuclease A, and 4 α-chymotrypsinogen A for 5 s at 50 mbar. Separation conditions: 20 kV, 25 °C, 215 nm.

In their study of a series of mono- and divalent metal cations, including calcium and magnesium, and zinc Binder et al. [37] found zinc to possess the strongest effect on the lipid phase behavior, followed by calcium. In view of that result, they proceeded to study the interaction of zinc with the PC coating. Zinc is a divalent ion but also a transition metal. Since zinc precipitates at pH 7.4 as $Zn(OH)_2$, the experiments were carried out with HEPES at pH 6.5 (HEPES pK_a ~ 7.5). Figure 16 (Figure 6 in paper II) shows a comparison of Zn^{2+} with Ca^{2+} and Mg^{2+} where a fused silica capillary was coated with PC/metal ion (molar ratio 1:20) and flushed between runs with the liposome dispersion. A protein sample was injected and the average plate numbers for the proteins were calculated.

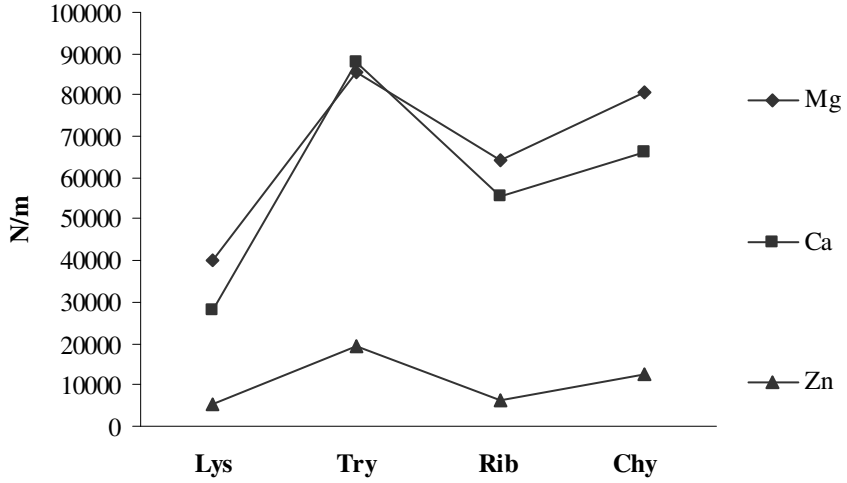


Figure 16. Plate numbers for proteins separated on a fused silica capillary (30/37 cm, ID/OD 50/375 µm) coated with POPC and Zn^{2+}, Ca^{2+}, or Mg^{2+}. PC/metal ion molar ratio of 1:20, in 40 mM HEPES at pH 6.5. The protein sample was injected for 5 s at 50 mbar and the separation was made at 25 °C at 20 kV with detection at 215 nm.

The plate numbers for the proteins were lower at pH 6.5 than at pH 7.4 (cf. Figure 14). The plate numbers at pH 6.5 were similar in the presence of calcium and magnesium, but considerably lower in the presence of zinc, probably due to interactions between zinc and the proteins. The migration times of the proteins were similar for zinc, calcium, and magnesium (data not shown). The RSDs for the migration times were less than 1% when coating was done with PC and Zn^{2+}, Ca^{2+}, or Mg^{2+}. Even though the plate numbers were worse when zinc was added, the stability of the coating was as good for zinc as for the other metal ions.

6.2.3 Addition of cetyltrimethylammonium bromide

At low concentrations (one-tenth or less of the CMC), alkyltrimethylammonium salts have a stabilizing effect on phospholipid membranes [44, 48, 49]. In this work, the concentrations of cetyltrimethylammonium bromide (CTAB) were chosen to be below one-tenth of the CMC [III]. The influence of CTAB on eggPC capillary coatings was investigated in the presence and absence of Ca^{2+} ion. CTAB was added only to the liposome dispersion itself and was not present in the BGE solution.

First, test was made of the effect of adding 3.8 μmol CTAB to the eggPC dispersion (equivalent to 0.5 mol% CTAB/eggPC) before extrusion. The resulting phospholipid coating suppressed the EOF very poorly. Evidently, adding CTAB to the liposome dispersion before extrusion changes the structure of the phospholipid bilayer in a way that disfavors its binding to the capillary wall.

As a next attempt to improve the stability, CTAB was added to the phospholipid dispersion after extrusion. Four concentrations (0, 1.9, 3.8, and 5.6 μmol) were tested. Between runs the capillary was flushed for 1 min with the coating solution and then for 1 min with the BGE solution to remove unbound liposomes. The initial EOF mobilities and the changes in EOF during six consecutive runs at each concentration are presented in Table 4 (Table 1 in paper III).

Table 4. Effect of CTAB concentration on the EOF and on the stability of eggPC coating. CTAB was added to the coating solution after extrusion, and the capillary was flushed with liposome dispersion for 1 min between runs.

CTAB (μM)	Initial mobility (10^{-5} $cm^2V^{-1}s^{-1}$)	Change during six consecutive runs (%)
0	8.6	5.33
0*	5.5	8.04
1.9	5.3	4.97
3.8	3.5	7.34
5.6	2.8	8.90
3.8*	6.9	4.02

* 2.25 mM of $CaCl_2$ was added to the liposome dispersion.

As can be seen, a low concentration of CTAB (1.9 μmol) had a slight stabilizing effect on the phospholipid coating. However, increasing the amount of CTAB (≥ 3.8 μmol)

did not further stabilize the phospholipid coating, even though the EOF was more suppressed (relative to plain eggPC coating). At higher CTAB concentrations the stability of the coating decreased, which indicates slight destabilization of the phospholipid membrane due to increased charge repulsion. The overall performance (in terms of baseline noise and disturbances) was improved.

Experiments at the above-mentioned CTAB concentrations were also carried out without a liposome flush between the runs, but this caused the EOF to increase still more during the consecutive runs. As a next step both CTAB and Ca^{2+} were added to the coating solution [III]. The stability of the coating was similar to that reported in section 6.2.2 where only Ca^{2+} was added to the coating solution. Together the results demonstrate that adding CTAB to the liposome dispersion improves the stability of the capillary coating only at very low CTAB concentrations, and even then only to a small degree. Clearly, a quick flush of the capillary with the coating solution between runs is necessary. The reason why addition of CTAB to the liposome dispersion (before or after extrusion) did not improve the stability of the phospholipid coating may be the lack of CTAB in the BGE solution. In all previous studies that have claimed a stabilizing effect of low CTAB concentrations on phospholipid vesicles and solid–supported phospholipid bilayers, the CTAB was also present in the surrounding solution [44, 48, 49]. Our aim, however, was to have the BGE solution free from the coating material.

In an attempt to decrease the electrostatic interaction between the zwitterionic PC and the basic proteins and so increase the peak efficiency and performance of the analysis, we added small amounts of CTAB to the coating solution after extrusion of the liposome dispersion [III]. The overall performance of the protein separation was improved by adding CTAB to the coating solution. The baseline became more regular, even though some irregularities occurred from time to time, due to changes in the coating. The signal-to-noise ratio as well as the peak efficiency for lysozyme and α-chymotrypsinogen A, were better than for the 100% eggPC coating. The increase in peak efficiency revealed that interactions between the proteins and the phospholipid coating were suppressed.

The ability of CTAB to suppress and even reverse the EOF in fused silica capillaries has been reported by several groups [84, 127, 128]. In the present work, CTAB may both modify the phospholipid coating and interact with residual silanol groups on the capillary wall. At CTAB concentrations higher than 15 μM, the migration times of the proteins became unreasonably long and the repeatability of the separation decreased dramatically (the RSDs for migration times and peak heights increased by over 20% for ribonuclease A and α-chymotrypsinogen A). The effect of CTAB on the phospholipid coating is not straightforward. On the one hand CTAB influences the interactions between proteins and the phospholipid membrane, improving the signal-to-noise ratio and peak efficiency and on the other hand it decreases the stability of the coating.

6.2.4 Adjustment of temperature

Depending on the temperature of the environment, phospholipids exist in gel or fluid state. The phase transition behavior of liposomes has been reviewed by Taylor and Morris [129]. In the gel state, the hydrocarbon chains are fully extended and in trans conformation, and the polar head groups are relatively immobile. When the system is heated the phospholipids undergo transition to the fluid state, where the polar head groups are more mobile and the hydrocarbon chains are disordered ue to changes from all trans to trans and gauche conformations of the carbon bonds. The transition temperature depends on the nature of the polar head group and on the length and degree of saturation of the hydrocarbon chains [129]. The transition temperature increases with the degree of saturation and length of the hydrocarbon chain. Thus, DPPC, which is a fully hydrogenated long chain PC, has a higher T_m (41 °C) than POPC (-2 °C) or eggPC (-5 °C to -15 °C) [130].

Studies on the structure of phospholipid bilayers have shown that temperature strongly affects the bilayer thickness, the water content between bilayers, and the rigidity of bilayers [131]. Above T_m the bilayer thickness decreases due to shortening of the effective length of the hydrocarbon chains. At the same time the entropic motion of the chains increases causing the lateral area per lipid to increase with temperature [131].

6.2.4.1 Differential scanning calorimetric studies

The surrounding temperature determines whether the phospholipid bilayer is in gel or fluid phase. The lipids also exist in other phases than the lamellar phase: for example, the ripple and inverted hexagonal phases. Whether the lamellar bilayer is in gel or fluid phase has a great impact on the coating stability and separations in CE. As noted above, with DPPC in the more rigid gel phase, the EOF is suppressed and the coating is highly stable. When liposomes are prepared by extrusion, as here, it is essential to keep the temperature of the lipid dispersion above the transition temperature (T_m). Differential scanning calorimetric studies were carried out to determine the T_m of the liposome dispersions. For the studies the extruded liposome dispersions were diluted with 40 mM HEPES (pH 7.4) to a total lipid concentration of 0.4–0.5 mM. Representative HSDSC heating scans of DPPC/chol (up to 30 mol%) mixtures are shown in Figure 17.

Pure DPPC LUVs showed a sharp peak at 41.5 °C with an enthalpy change (ΔH) of 42.7 $kJmol^{-1}$. A small pre-transition peak was observed at about 35 °C (not visible in Figure 17). When cholesterol was added to the DPPC dispersion the heat capacity peak became broader and the ΔH smaller. The main transition peaks of PC/chol and SM/chol bilayers displayed two superimposed heat capacity peaks [132, 133]. The sharp endothermic component results from melting of the cholesterol-poor and the broad component from the cholesterol-rich membrane domains. In PC MLVs, the pre-transition has been shown to decrease with increasing amount of cholesterol [17].

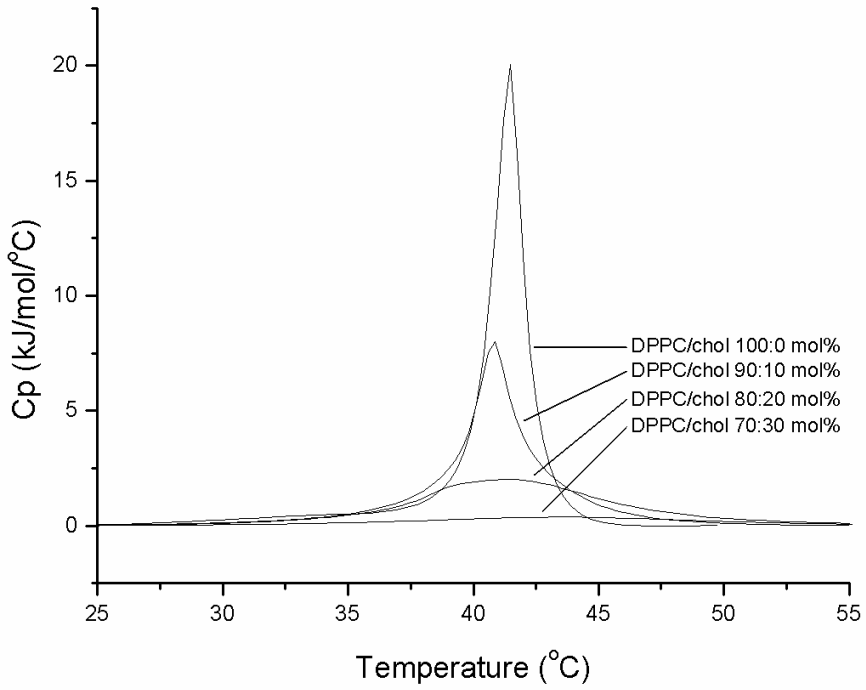


Figure 17. Microcalorimetric endotherms of DPPC/chol mixtures as a function of cholesterol content. Total lipid concentration of 0.5 mM in 40 mM HEPES, pH 7.4.

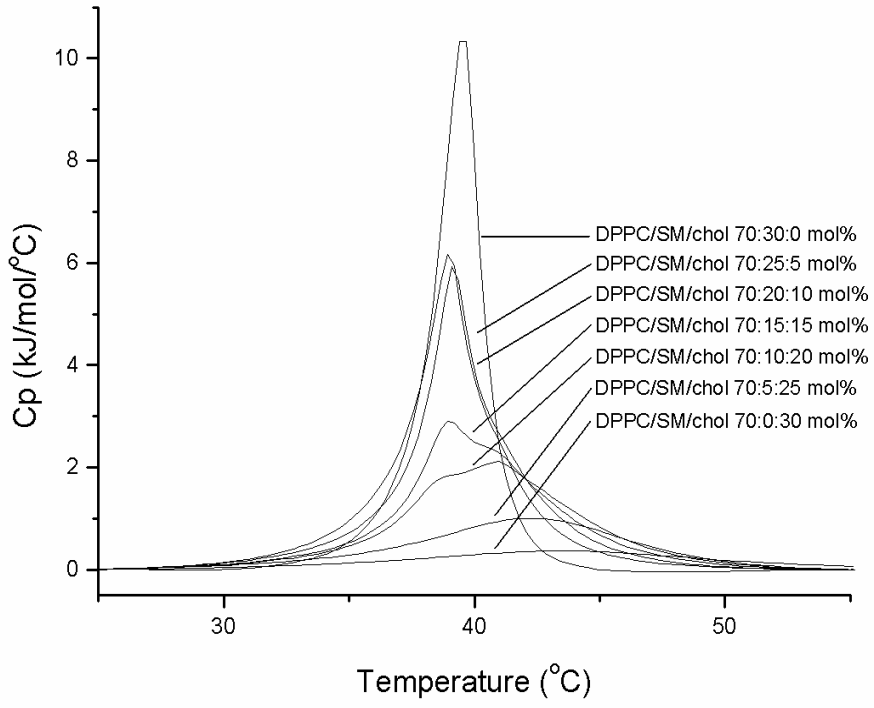


Figure 18. Microcalorimetric endotherms of DPPC/SM/chol mixtures as a function of SM and cholesterol content. Total lipid concentration of 0.4 mM (0.5 mM for 70:0:30 mol%) in 40 mM HEPES, pH 7.4.

As a move toward more natural membranes, binary and ternary mixtures of DPPC, SM, and cholesterol (Figure 18, Figure 1 in paper IV) were studied. The DPPC concentration

was kept constant at 70 mol%, while the amounts of SM and cholesterol were varied between 0 and 30 mol%. Increasing the cholesterol content in DPPC/SM/chol LUVs broadened the heat capacity peak, and at 30 mol% of cholesterol the peak was barely detectable. In addition, as the content of cholesterol was increased the main phase transition shifted toward higher temperatures (Figure 17). The binary and ternary mixtures of DPPC with SM and cholesterol behaved rather differently at higher cholesterol contents. For example, with 20 mol% cholesterol the heat capacity curves of DPPC/chol 80:20 mol% (Figure 17) and DPPC/SM/chol 70:10:20 mol% (Figure 18) were distinctly dissimilar. In contrast to the binary DPPC/chol mixtures, the ternary complexes showed two partly overlapping endothermic reactions (Figure 18). This is likely to be a consequence of the formation of cholesterol-poor and cholesterol-rich microdomains.

Table 5 (Table 1 in paper IV) lists the enthalpies (ΔH), half widths ($T_{1/2}$), and maximums of the heat capacity curves (T_m) shown in Figures 17 and 18. At higher mole fractions of cholesterol, the enthalpies for the liposome dispersions decreased while the half widths increased. The enthalpies were also calculated in proportion to the total amount of phospholipid (cholesterol excluded) and in proportion to the amount of DPPC present in the liposome dispersion. The decrease in enthalpy was thereby confirmed to be due to cholesterol interaction with the bilayer and not to the decreasing phospholipid (DPPC and SM) or DPPC percentage.

Table 5. The T_m, $T_{1/2}$, and ΔH values for DPPC/SM/chol liposome dispersions measured by HSDSC. Experimental conditions: response time 5.6 s, scans 10-60 °C at a heating rate of 60 °C h^{-1}. Before each scan, the sample (0.4–0.5 mM) was kept at 10 °C for 30 min. Runs were made twice for each dispersion and the values listed are for the second run.

DPPC/SM/chol	T_m (°C)	$T_{1/2}$ (°C)	ΔH(kJ/mol)[a]	ΔH(kJ/$mol_{p\text{-}lipid}$)[b]	ΔH(kJ/mol_{DPPC})[c]
100:0:0 mol%	41.5	1.6	42.7		42.7
90:0:10 mol%	40.9	2.1	29.8		33.1
80:0:20 mol%	41.4	8.0	20.3		25.4
70:0:30 mol%	43.7	38.2	5.8		8.3
70:30:0 mol%	39.6	2.1	28.0	28.0	39.9
70:25:5 mol%	38.9	2.9	26.8	28.2	38.2
70:20:10 mol%	39.1	2.7	25.6	28.4	36.6
70:15:15 mol%	38.9	5.3	19.6	23.0	28.0
70:10:20 mol%	41.0	7.2	17.7	22.1	25.2
70:5:25 mol%	42.2	9.3	10.7	14.2	15.2

[a] Calculated on the basis of total amount of lipids, SM, DPPC, and cholesterol

[b] Calculated on the basis of total amount of phospholipids, SM, and DPPC

[c] Calculated on the basis of total amount of DPPC

The calorimetric studies affirm that adding cholesterol to a liposome dispersion of DPPC or of DPPC and SM broadens the main phase transition and eliminates it at higher cholesterol concentrations (≥ 30 mol%). These DSC results are in good accord with the results of other groups [13, 132,134-136]. Anderson and McConnell [136] developed a thermodynamic model describing the reaction between cholesterol and

phospholipids to form a condensed complex. In their studies, pure DPPC dispersions showed a sharp peak at 41 °C, but with increasing amount of cholesterol, the endothermic transition became weaker until, at ≥ 25% cholesterol, it disappeared. A second, broad transition became visible at higher temperature at 10% cholesterol. The sharp peak is due to the chain melting of pure phospholipid. Figure 18 reveals an interesting phenomenon in the curves obtained with DPPC/SM/chol ratios of 70:15:15 mol% and 70:10:20 mol%. Apparently, there are two, partly overlapping endothermic transitions. This might be due to phase separation in the ternary liposome mixture. The phase separation might be interesting if the domains were to behave differently in lipid–lipid or lipid–protein interactions. Relevant to this, domains enriched in certain types of lipids, such as sphingolipids and cholesterol, are believed to function as platforms for cellular signaling transduction. If so, these domains would interact with only certain types of proteins or peptides. Accordingly, the method developed here may be suitable for defining the interacting partners in heterogeneous lipid assemblies.

Spink et al. [137] observed that the decrease in the temperature of the heat capacity peak maximum with addition of cholesterol is nonlinear. At higher concentration the nonlinear decrease might be due to the second, broad component of the transition. The sharp main transition peak from the acyl chain melting disappears at concentration of 20-25 mol% cholesterol, allowing the second, broader peak to dominate in the heat capacity curve [17]. This is probably the main reason why in Figure 17 the T_m seems to increase for DPPC/chol dispersions at higher amount of cholesterol, rather than to decrease as was expected from the results of Anderson et al. [136] and Spink et al. [137]. The enthalpies for the liposome dispersions at higher mole fractions of cholesterol decreased, as also observed by others [17]. The decrease in enthalpy is due to cholesterol causing fluidization (disordering) of the hydrocarbon chains of PC in the gel phase and inhibiting the flexing of chains in the liquid-crystalline phase [13]. Cholesterol intercalates between the acyl chains and diminishes the chain–chain interactions [138].

6.2.4.2 Coating and running temperatures

The effect of the temperature-induced gel- to fluid-state transition on the coating stability was investigated by studying DPPC above and below its T_m (41°C). Puu and Gustafson [28] found DPPC to exhibit much better fusion than POPC when proteoliposomes containing 50% of DPPC or POPC were transferred onto platinum or silicon supports. They suggested that nonperfect liposomes, prepared under their transition temperature, might more easily form planar lipid membranes than liposomes prepared at higher temperature. The capillary was coated as described in the experimental section (Chapter 5). After coating, the capillary was flushed for 2 min with BGE solution to remove unbound liposomes. Before the runs were started, a 20-min period was allowed for heating (from 25 °C to 45 °C) or cooling (from 45 °C to 25 °C) the capillary. Four different systems were investigated: working at 1) 25 °C or 2) 45 °C during both coating and runs, 3) coating at 25 °C with runs made at 45 °C, and 4) coating at 45 °C with runs made at 25 °C. Between the runs the capillary was flushed

with BGE solution for 2 min. The stabilities of these four systems during runs are presented in Figure 19.

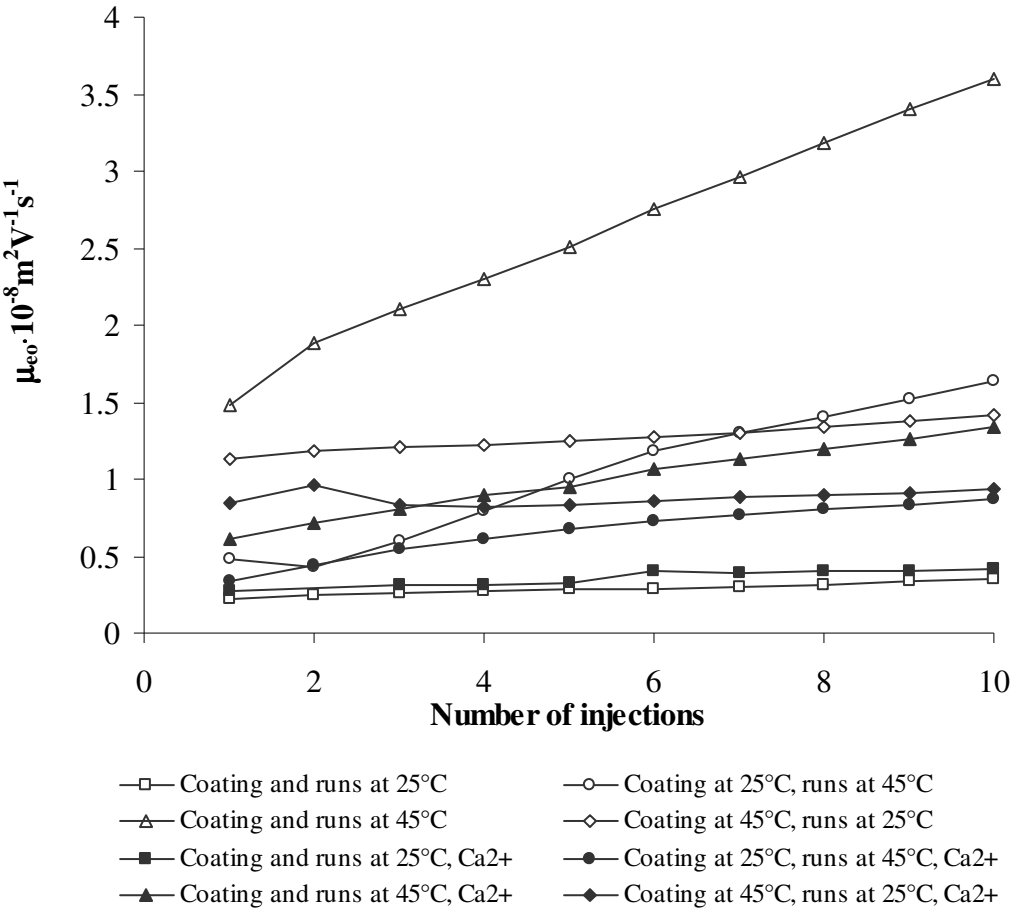


Figure 19. Effect of temperature on stability of DPPC coating with and without calcium. Coating and running temperatures were as follows: symbols on left; coating and runs at 25 °C or 45 °C; symbols on right: coating at 25 °C and runs at 45 °C, coating at 45 °C and runs at 25 °C. 40 mM HEPES at pH 7.4 was used as BGE solution. Injection of methanol for 2 s at 50 mbar. Separation conditions: voltage 20 kV, UV detection 200 nm

As seen from Figure 19, the coating is most stable and the EOF most strongly suppressed when both coating and runs are done at 25 °C. The EOF is much less suppressed with coating and runs at 45 °C, and it increases with each injection evidently owing to some leak of lipids out of the capillary. Coating at 25 °C with runs at 45 °C shows an initial suppression of the EOF to almost the same level as when both coating and runs are made at 25 °C. With continued heating at 45 °C, however, there is a similar trend as for coating and heating at 45 °C because the liposomes are transferred to their fluid state and leak out of the capillary. With the fourth system (coating at 45 °C, runs at 25 °C) the EOF is less suppressed, but after cooling of the capillary to 25 °C the EOF is stable and the liposomes do not leak out of the capillary because they are in the gel state.

In addition, the effect of calcium on the DPPC coating was studied. The same four systems were used but with the addition of Ca^{2+} to the DPPC dispersion (molar ratio 1:3

for DPPC/Ca^{2+}) (Figure 19). Adding calcium generally improved the coating by lowering and stabilizing the EOF. For the system where both coating and runs were made at 25 °C, however, calcium did not affect the coating, suggesting that working in the gel state does not require the addition of calcium. The mean electroosmotic mobility of 10 runs with DPPC was ~ $0.3 \cdot 10^{-8}$ $m^2s^{-1}V^{-1}$, whereas for both PC/Ca^{2+} (1:3) and for PC/Mg^{2+} (1:3) it was ~ $0.6 \cdot 10^{-8}$ $m^2s^{-1}V^{-1}$. The coating is clearly better when the liposomes are in the gel state because the liposomes are then more rigid.

6.2.5 Addition of cholesterol

Cholesterol minimizes the movement of the acyl chains in the bilayer by changing the amount of trans-gauche isomers, as discussed in section 6.2.4.1, and it makes the bilayer more rigid and gel-like [130]. Thus, a logical next step was to investigate how cholesterol affects the stability of a DPPC/SM coating on fused silica. The effect of cholesterol (0-30 mol%) on the stability of DPPC coatings is shown in Figure 20. In addition, the effect of working at different temperatures was studied for the DPPC/SM/chol coatings. [IV] The coatings and runs were made at 25 °C and 45 °C, i.e., with the phospholipid phase in gel and fluid phases, respectively.

Although a mixture of DPPC and cholesterol is often used as a model bilayer for natural membranes, the composition is significantly different from the phospholipid and fatty acid composition of natural membranes. As many as 500-1000 lipid species are present in a natural membrane [139]. Although a bilayer consisting of DPPC, SM, and cholesterol is thus a gross simplification of a natural membrane, it gives a good idea of how membrane-like bilayers might coat a capillary.

The DPPC coating in the gel phase (25 °C) is already highly stable without cholesterol (Figure 19). Adding up to 30 mol% cholesterol does not affect the coating stability. The EOF is slightly faster for the cholesterol-containing coating than the pure DPPC coating, but the EOF (DMSO as marker) is still below 1×10^{-8} $m^2V^{-1}s^{-1}$, and the free silanols on the inner surface of the silica capillary are evidently well shielded by the coating. For runs at 45 °C the EOF of the DPPC coating increases from 1.5×10^{-8} $m^2V^{-1}s^{-1}$ to 3.5×10^{-8} $m^2V^{-1}s^{-1}$ over ten runs, suggesting that the coating is slowly leaking out of the capillary. When 10 or 20 mol% cholesterol is added to the DPPC dispersion in fluid phase, the EOF only increases by 1.5 units over 10 runs. With 30 mol% cholesterol the EOF is suppressed to about 3.5×10^{-8} $m^2V^{-1}s^{-1}$ in the first run, but the increase in the EOF over 10 runs is less than 0.5 units. Since even here the EOF is lower than for the uncoated capillary (6.7×10^{-8} $m^2V^{-1}s^{-1}$ at 45 °C), the silanol groups must be shielded by the phospholipid coating. The stability of the EOF is probably due to a change in the physical properties of the lipid coating.

For comparison, results for POPC 100 mol%, POPC/chol 70:30 mol%, and POPC/DPPC 50:50 mol% coatings at 25 °C are included in Figure 20 (Figure 2 in paper IV). At 25 °C POPC is in the fluid phase, and the coating stability is improved by addition of 30 mol% cholesterol. The DPPC/chol 70:30 mol% coating (45 °C) is comparable with the POPC/chol 70:30 mol% coating (25 °C) taking into account the

faster EOF at higher temperature (4.9×10^{-8} $m^2V^{-1}s^{-1}$ at 25 °C; 6.7×10^{-8} $m^2V^{-1}s^{-1}$ at 45 °C) due to the lower viscosity of the BGE solution. The POPC/DPPC 50:50 mol% coating is highly stable. The EOF of the POPC/DPPC 50:50 mol% coating is faster than that of the pure DPPC coating, but the stability is improved over that of the pure POPC coating. Protein separations were also carried out on DPPC-coated capillaries. A sample of lysozyme, ribonuclease A, and α-chymotrypsinogen A was injected to a capillary coated with DPPC in 40 mM HEPES at pH 7.4. The RSDs for the migration times of the proteins on the DPPC-coated capillary were less than 1%, and the plate numbers were max. 100 000 m^{-1}.

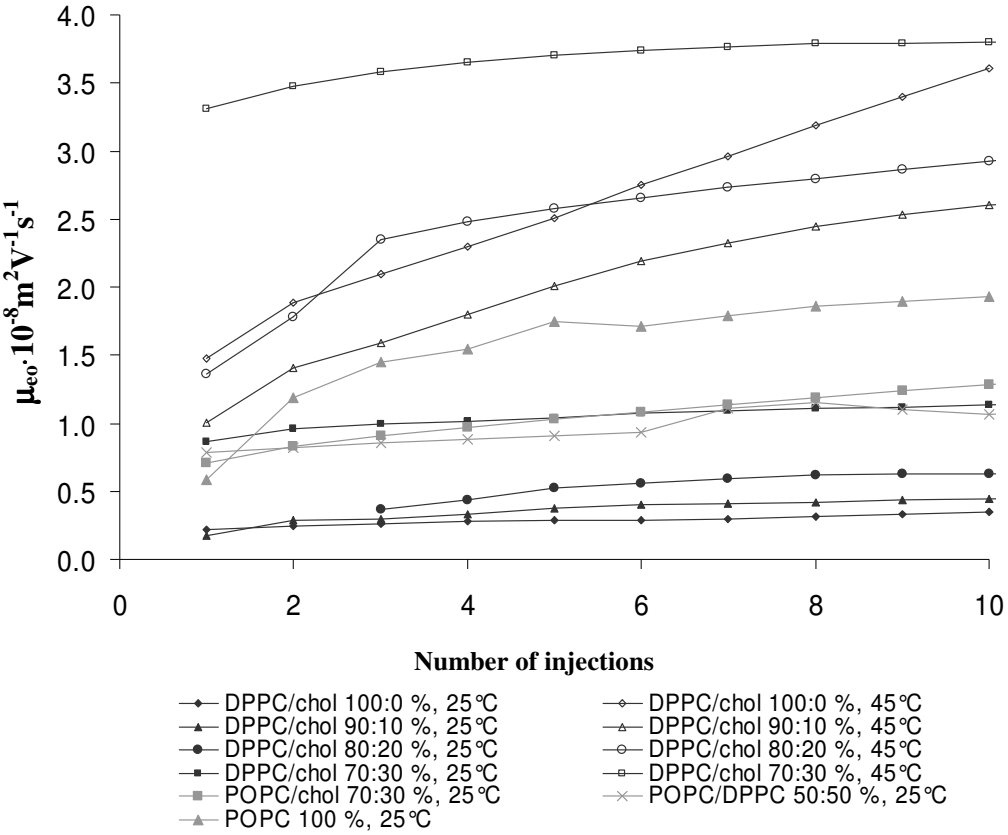


Figure 20. Effect of increasing of molar percentage (0-30 mol%) cholesterol on the EOF of the DPPC coating at 25 °C and 45 °C. For comparison, curves of the stability of a POPC 100 mol%, POPC/chol 70:30 mol%, and POPC/DPPC 50:50 mol% coatings are included in the figure. The fused silica capillary (30.0/38.5) cm was preconditioned and coated with 1 mM liposome dispersion (except for DPPC 100 mol% where the concentration was 0.75 mM). Injection of 0.05 % DMSO for 2 s at 50 mbar. Separation conditions: 20 kV, 25 °C or 45 °C, 200 nm.

Next, the coating stability was studied for a ternary mixture of DPPC, SM, and cholesterol (see Figure 21, Figure 3 in paper IV). The pure DPPC coating still has the lowest electroosmotic mobility. Although the electroosmotic mobility is significantly higher for the coatings with SM 100 mol% and SM/chol 70:30 mol% than for the rest of the coatings, the suppressed EOF suggests that the capillary remains coated. Adding 30 mol% SM to a DPPC coating increases the electroosmotic mobility, but the EOF is still suppressed and the coating is stable. The stability of the coating is preserved with increasing amounts of cholesterol in place of SM, but no trend is apparent in the

electroosmotic mobility. The small differences in mobility for the different liposome mixtures are probably due to normal variation from one coating to the next.

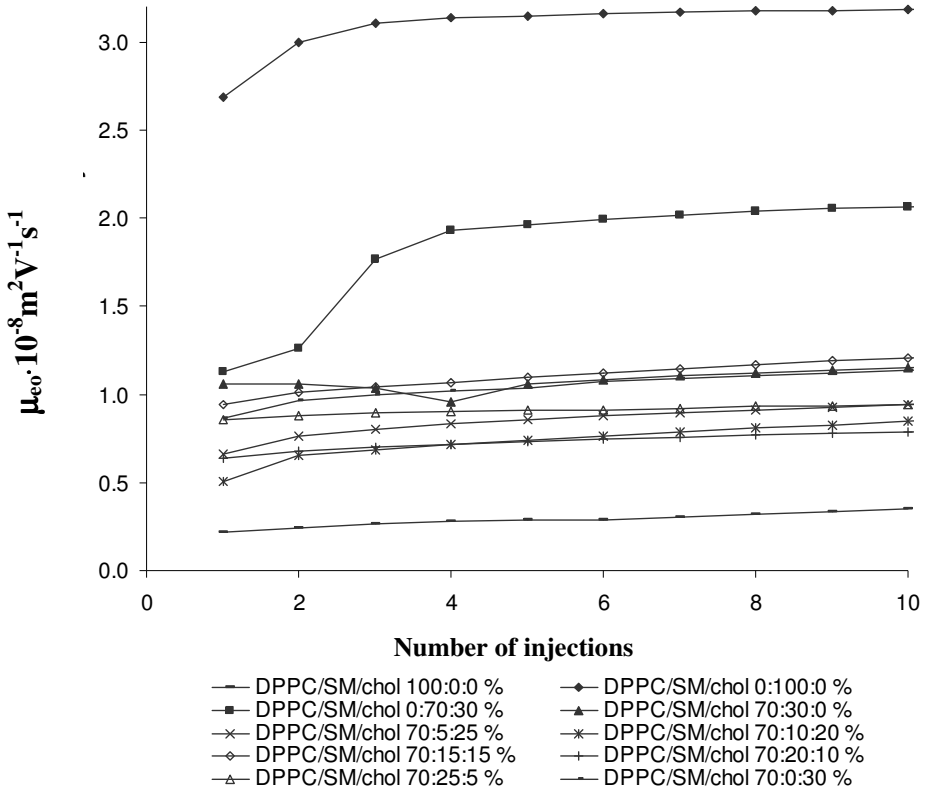


Figure 21. Effect of cholesterol on the EOF in DPPC- and SM-coated capillaries. The fused silica capillary (30.0/38.5) cm was preconditioned and coated with 1 mM liposome dispersion as described in the experimental section. Injection of DMSO for 2 s at 50 mbar. Separation conditions: 20 kV, 25 °C, 200 nm.

I wished to know if there is a range where the coating stability is improved by favorable interaction of cholesterol with SM or DPPC. As already noted, cholesterol preferentially interacts with saturated PCs [22], and therefore it favorably interacts and forms a liquid-ordered phase upon mixing with DPPC, as well as with the structurally similar SM [140, 141]. The optimal carbon chain length for cholesterol to hydrophobically match the PC acyl chains in the bilayer was found by McMullen et al. [18] to be 17 or 18. At shorter chain lengths, they suggest, the gel phase is stabilized by cholesterol. Guo et al. [27] compared the interaction of cholesterol with SM and DPPC by solid-state NMR spectroscopy and found that the interactions are generally similar, owing to the similarity of the fatty chains of SM and DPPC. They also proposed that cholesterol might interact specifically with SM in a natural membrane because the degree of unsaturation is lower for SMs in a natural membrane than for PCs.

The results (Figure 21) indicate that liposomes prepared with various amounts of cholesterol and SM (up to 30 mol% total) added to a DPPC dispersion can provide a highly stable coating on a fused silica capillary. Since the EOF is considerably suppressed, the coverage of the phospholipid coating on the silica surface is good. The

structure and role of membrane rafts in cell functions have been studied extensively, as reviewed in refs. 25, 26, 142. The rafts in cell membranes are probably liquid-ordered-like domains, which are intermediate between the gel and the liquid-crystalline phase [143]. For mixtures of cholesterol and phospholipids, the liquid-ordered-like domain emerges at cholesterol concentrations above 25% [136]. The results demonstrate that adding up to 30 mol% cholesterol, i.e., making the coating solution more similar to a natural membrane, does not disturb the bilayer formation or the coating stability at 25 °C. The findings suggest the suitability of this kind of coating as a model for studying membrane–analyte interactions. In runs at 45 °C, the EOF of the DPPC 100% coating increased when several injections were made to the same capillary, suggesting that, the increase in fluidity caused the coating to leak out of the capillary. This increase in EOF was expected since the rigidity of the coating is important in maintaining its stability in the capillary. Clearly some kind of PC base for the coating solution is needed, and, indeed, natural plasma membranes have PC as a major component.

6.2.6 Addition of polyethylene glycol phospholipids

PEGylated lipid aggregates are a promising new class of membranes for drug-delivery. The PEG polymer stabilizes the lipid carrier and prolongs the residence time in the blood circulation. In study V the suitability of PEG aggregate as coating material for CE was investigated as well as the differences in properties (size, surface charge, and hydrophobicity) of the PEG lipids. Commercial phospholipids with covalently attached PEG chains were added to POPC liposomes. The PEG lipids consisted of PEG of different molar masses (1000, 2000, and 3000) conjugated to phospholipids DMPE (C14) of DSPE (C18).

6.2.6.1 Sizes and zeta potentials

A comparison of the sizes of PEGylated vesicles was carried out with DSPE-PEG (molar masses 1000, 2000, and 3000) and POPC/DSPE-PEG molar ratio of 80:20 mol%. The average diameters were measured by AsFlFFF and DLS (633 nm) (Table 6). The POPC/DSPE-PEG3000 dispersion was additionally measured at 488 nm, and gave a diameter of 76±6.5 nm (n=3 and the polydispersity index was 0.175–0.194). In general, the vesicle sizes decreased as PEGs with higher molar mass were attached. This decrease in size was expected to occur, owing to steric repulsion among the PEG chains [144]. AsFlFFF revealed two peaks for the POPC/DSPE-PEG2000 sample but DLS only one peak. Clearly, there is an advantage to using a separation method (AsFlFFF) in the determination of heterogeneous particle solutions. The AsFlFFF results suggest that both liposomes and discoidal micelles are present in the POPC/DSPE-PEG 2000 dispersion, and this finding was confirmed by EM (Figure 2 in Paper V). The presence of several different structures in DSPE-PEG2000 preparations was recently demonstrated [145]. Investigation by EM indicated that the POPC/DSPE-PEG3000 sample was dominated by discoidal micelles (Figure 2 in Paper V). The predominance of discoidal micelles over liposomes thus increased with increase in the PEG chain length, while the average size of the vesicles decreased. For comparison, the sizes of

POPC/DMPE-PEG3000 aggregates (DMPE-PEG has a shorter acyl chain than DSPE-PEG) were determined by DLS. Unexpectedly, the results (Table 6, Table 1 in paper V) showed that there was no shrinkage of the liposomes, despite the high molar mass of the DMPE-PEG3000 lipid. Evidently, the acyl chains of the PEG lipid affect the size of the liposomes.

Table 6. Sizes measured by AsFlFFF, DLS at 633 nm (size by volume), and AFM, and zeta potentials of POPC (100 mol%) and 80:20 mol% POPC/PEGylated aggregates.

Lipid dispersion	AsFlFFF (nm)	DLS (nm)	AFM (nm)	Zeta potential (mV)
POPC	98 ± 3.0	98 ± 3.0		-18.7 ± 2.0
POPC/DSPE-PEG1000	108 ± 8.5	79 ± 1.1	84 ± 32	-16.1 ± 1.2
POPC/DSPE-PEG2000	59 ± 8.4; 21 ± 15.8	69 ± 5.9	56 ± 15	-9.4 ± 0.8
POPC/DSPE-PEG3000	65 ± 6.0	53 ± 6.2	45 ± 10	-3.9 ± 0.7
POPC/DMPE-PEG3000		104.1 ± 2.1		-13.1 ± 2.6

The electrical surface potential of PEGylated liposomes is the electrical potential determined in the plane of the negatively charged phosphate group of the phospholipid. The zeta potential, on the other hand, is the electrokinetic potential determined farther away from the actual lipid headgroup plane (where the electrostatic potential equals the zeta potential) and closer to the boundary between the fixed and mobile electrical parts of the Stern double layer. The zeta potential is therefore a function of the surface charge of the particle and the nature and composition of the surrounding suspension medium.

Zeta potentials were measured to obtain a better understanding of the PEGylated aggregates. The data in Table 6 show a clear difference between the absolute values of the zeta potentials of the PEGylated aggregates and the value obtained for pure POPC vesicles (-18.7 ± 2.0 mV). The higher the molar mass of PEG (i.e., the longer the PEG chain), the lower is the surface charge of the vesicles. This can be explained in terms of shielding of the negatively charged vesicle surface by the exposed PEG chains, which decreases the zeta potential of the vesicle [146, 147]. However, the zeta potential of the POPC/DMPE-PEG3000 aggregates was about -13 mV, which shows that the shorter C14 chains of the DMPE-PEG lipid clearly affect the packing of the lipids in the liposome.

6.2.6.2 Aggregates as capillary coating material

Experiments aimed at optimizing the molar ratio of POPC to PEG lipids were carried out with DSPE-PEG3000. A new capillary was used for each liposome composition. Capillaries were coated with the PEGylated lipid dispersions, and 36 injections of the EOF marker were made to each capillary. Between runs there was only a short flush (2 min) with the BGE solution (Figure 22, Figure 3 in paper V).

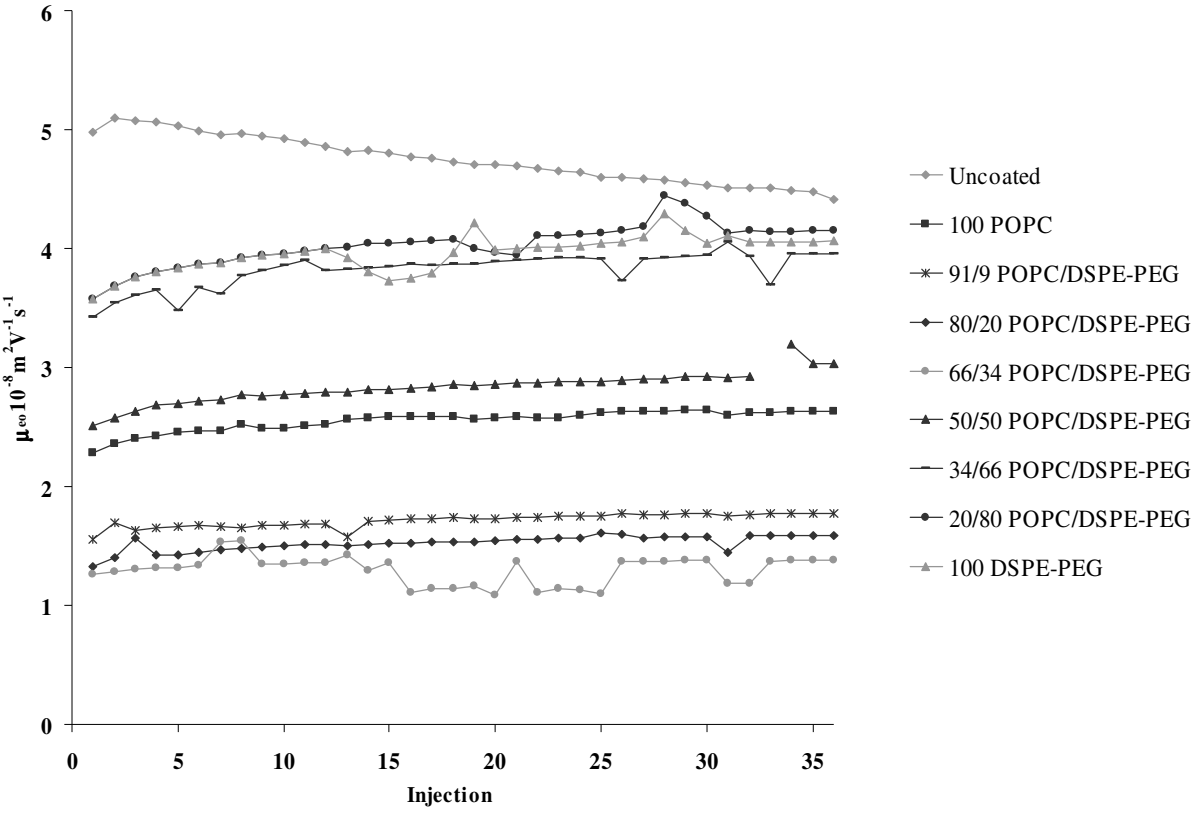


Figure 22. EOF values of POPC/DSPE-PEG3000 coatings of various molar ratios (100:0–0:100 mol%). Lipid solvent and BGE solution was HEPES pH 7.4 (ionic strength of 9 mM). Running conditions: capillary 23.5/32.0 cm; voltage 20 kV; temperature 25 °C; injection of EOF marker (DMSO sample) 2 s 50 mbar; UV detection at 200 nm.

As shown in Figure 22, highly stable coatings with strongly suppressed EOFs (~$1.5 \cdot 10^{-8}$ $m^2s^{-1}V^{-1}$) were obtained with 80:20 mol% and 91:9 mol% of POPC/DSPE-PEG3000 dispersions. Increasing the amount of DSPE-PEG3000 to 34 mol% suppressed the EOF slightly below that of the 80:20 composition, but the repeatability of the injection was poorer. With 50 mol% DSPE-PEG3000 in the dispersion, the EOF was stable but higher than with pure POPC. Higher portions (66–100 mol%) of DSPE–PEG3000 in the lipid dispersion decreased the coating stability.

Earlier studies have shown that there is a maximum amount of PEG lipid that can be included before structural destabilization of the liposome occurs. At concentrations above 10 mol% of DSPE-PEG2000, liposomes are disrupted into large bilayer disks [148, 149]. In addition, discoidal micelles are formed at low PEG-lipid concentrations (<5 mol%) in mixtures of DPPC or DSPC with PEG lipids [150], and when DSPE-PEG2000 was added to an extruded eggPC sample open bilayer aggregates appeared as an intermediate structure before micelle formation [151]. The acyl chains of PC are of importance, however, because when eggPC vesicles are mixed with DSPE-PEG2000, micelle formation occurs at PEG-lipid concentrations above 10 mol%. In general, an increase in the PEG-lipid concentration in the lipid mixture increases the lateral repulsive properties of the surface of the lipid bilayer through extensive hydration around the polar head group [152]. As a means to reduce the degree of repulsion, the

vesicles then contract while the curvature of the grafting surface increases. A change in the structure and surface properties of the vesicles with increasing amount of PEG-lipids, resulting in changed packing on silica, would partly explain the variations in EOF observed in this work. As mentioned above, a highly suppressed and stable EOF was achieved with the 80:20 mol% POPC/DSPE-PEG3000 dispersion, and this molar ratio was used in subsequent experiments.

In the next step we evaluated the effects on EOF of the acyl chains and molar mass of PEG in the PEGylated lipid. The results in Figure 23 (Figure 4 in paper V) show that the lowest EOF was obtained with the POPC/DSPE-PEG3000 coating and the next lowest with the POPC/DSPE-PEG2000 coating. All PEGylated dispersions gave stable coatings. The highest EOF values were for dispersions containing PEG1000 lipids. Inspection of the sizes of the different POPC/DSPE-PEG lipid aggregates (given in Table 6) shows that the POPC/DSPE-PEG1000 liposomes tended to be much larger than the corresponding PEG2000 and PEG3000 aggregates. Hence, one explanation of the higher EOF values with the POPC/DSPE-PEG1000 liposomes than with the two other lipid dispersions could be the larger size of the POPC/DSPE-PEG1000 aggregates, which leads to poorer packing and less effective shielding of the silanol groups on the capillary wall.

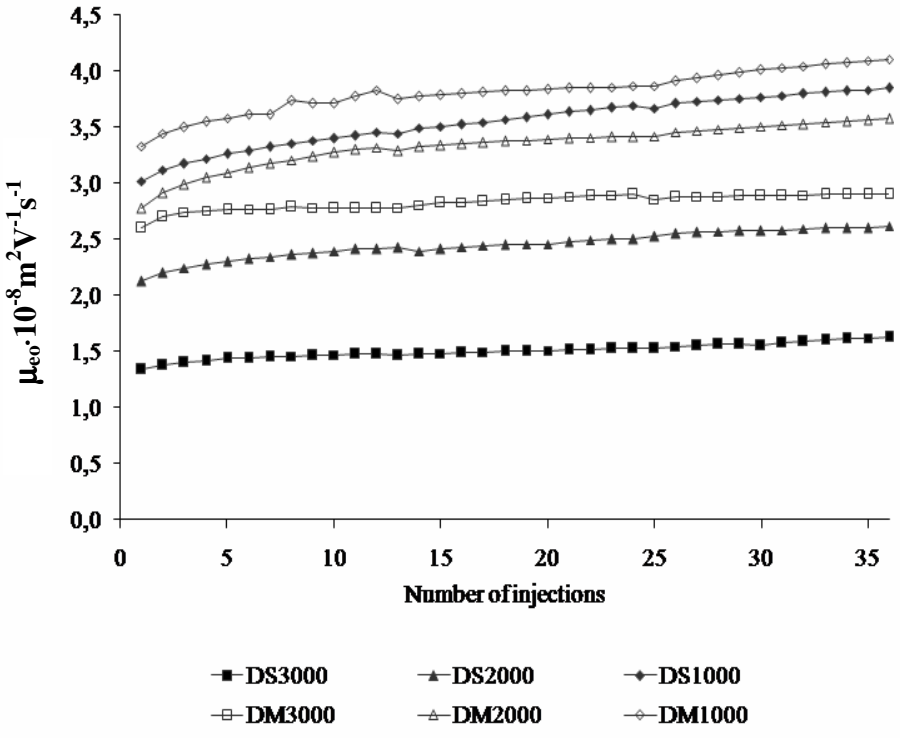


Figure 23. Influence of the acyl chain length and molar mass of PEG lipids on the formation of coatings for electrophoresis. The coating solutions comprised 1 mM 80:20 mol% POPC/PEG lipid in 20 mM HEPES pH 7.4. Running conditions as in Figure 22.

QCM and AFM measurements were carried out to test this hypothesis. In a study of negatively charged liposomes of sizes around 100 nm by QCM, Viitala et al. [111] found that, depending on the lipid and the composition of the buffer solution, two

distinct structures appeared: a rather rigid supported lipid bilayer (SLB) and a viscoelastic supported vesicle layer (SVL). The results of our QCM measurements of the adsorption of the PEGylated lipid aggregates onto silica showed an increase in the absolute frequency change, the standard deviation of normalized frequencies, the resistance, and the adsorbed mass with the size of the attached PEG. The increases in the standard deviation for the normalized frequencies and the resistance values with increasing PEG chain length indicate that, relative to other lipid aggregates, those with longer PEGylated chains adsorb in greater amount on the silica surface, and also form a more viscoelastic layer when they are adsorbed.

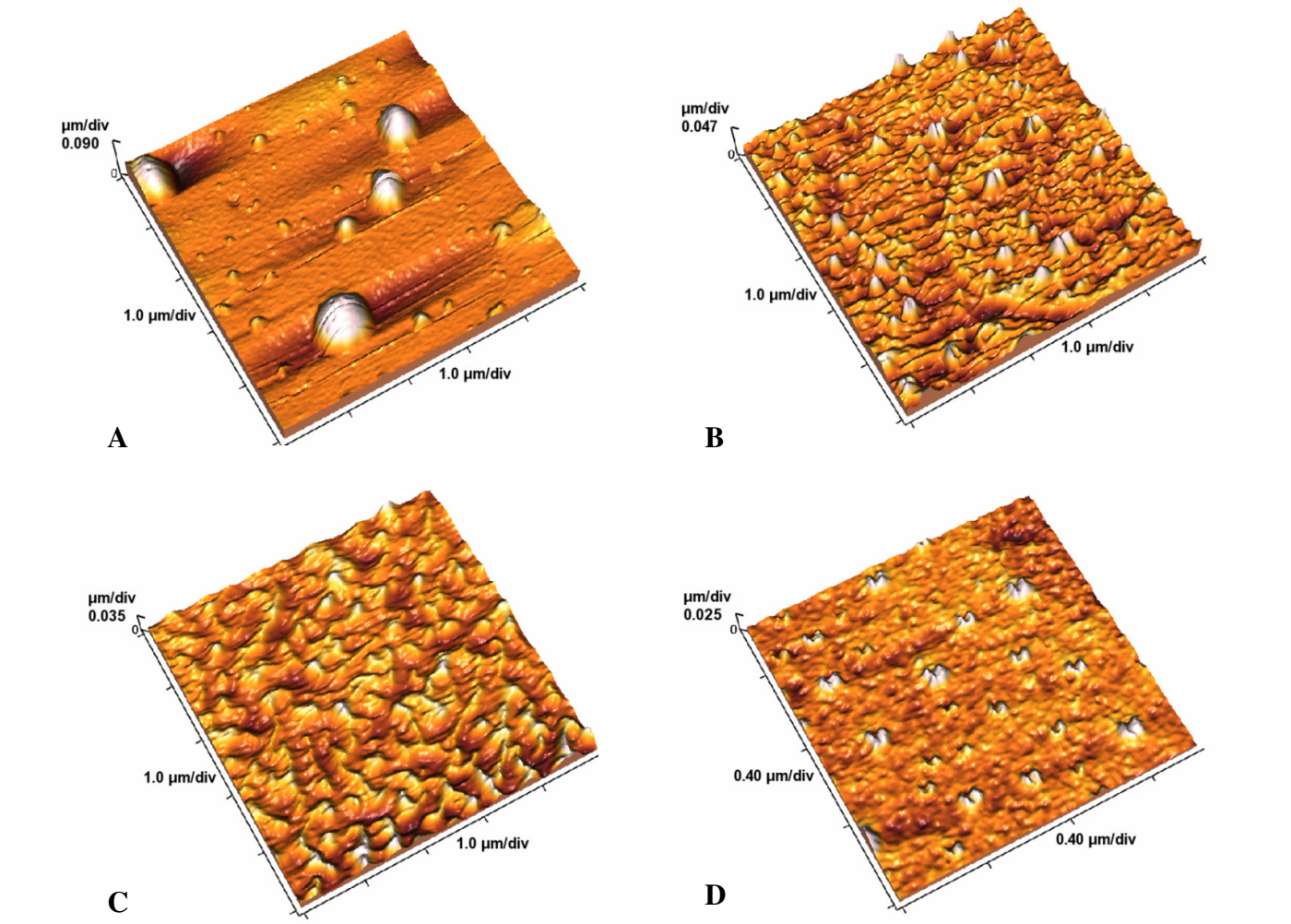


Figure 24. AFM images (5 µm × 5 µm) of PEGylated lipid aggregates of (A) POPC/DSPE-PEG1000, (B) POPC/DSPE-PEG2000, and (C) POPC/DSPE-PEG3000 coated in 1 mM concentrations on fused silica capillary outer walls. Figure (D) is a 1.6 µm × 1.6 µm image of a capillary wall covered with a 0.2 mM concentration of POPC/DSPE-PEG3000.

AFM studies were carried out on PEGylated lipid aggregates on the outer walls of fused silica capillaries. Variations can be seen (Figure 24, Figure 5 in paper V) in particle size and surface interaction, ranging from large disperse liposomes in A to smaller particles

showing evidence of monolayer formation in B, and a rough coating of lipid aggregates in C. A 1.6 μm × 1.6 μm image (D) of a capillary wall covered with a 0.2-mM concentration of POPC/DSPE-PEG3000 gave evidence of small discoidal micelles in a sparser coating of the surface. The sizes of the lipid aggregates determined by AFM corresponded well with the sizes obtained by the other techniques. The thicknesses of the disks were 9.4±2.79 nm.

Comparison of the effect of the acyl chains (C14 vs C18) in PEGylated lipids with the same PEG moiety showed that, in all cases, EOF values were lower with the DS (C18) conjugates. The effect was pronounced in the case of the PEG3000 lipid aggregates. Noteworthy was the lower EOF and, correspondingly, lower net negative charge of the POPC/DSPE-PEG2000 coating than of the POPC/DMPE-PEG3000 coating. Here, clearly other factors than simply the molar mass of the PEG lipid were playing a role. The zeta potential of the POPC/DSPE-PEG2000 aggregates was about -9 mV, whereas that of the POPC/DMPE-PEG3000 aggregates was about -13 mV. In addition, the POPC/DSPE-PEG2000 aggregates were smaller than the POPC/DMPE-PEG3000 aggregates. Hence, the lower net negative charge of the POPC/DSPE-PEG2000 coating than of the POPC/DMPE-PEG3000 coating was probably due to a combination of smaller size and lower charge of the POPC/DSPE-PEG2000 aggregates. As shown by AFM and QCM, the smaller the particles the better is the packing of aggregates and shielding of silanol groups on the silica wall (i.e., the lower is the EOF), and further, the lower the liposome surface charge, the lower is the EOF of the coating.

The shielding effect of the PEGylated lipids in the lipid aggregates against lipid–analyte interactions was studied (paper V) through injection of a mixture composed of low-molar-mass (MWs 266–360 g mol^{-1}) drugs atenolol, aldosterone, testosterone, and dichlorophenamide (UV detection). POPC/DSPE-PEG1000, POPC/DSPE-PEG2000, and POPC/DSPE-PEG3000 80:20 mol% lipid dispersions were used as coating material. Comparison of the effective electrophoretic mobilities of atenolol with the different coatings showed that there were no strong interactions of the positively charged analyte with the PEG-lipid membranes under these conditions (HEPES pH 7.4). Aldosterone, which is a steroid-based analyte with a log $P_{o/w}$ value of ca. 0.7, did not have strong hydrophobic interactions with any of the membranes. With testosterone, which is the most hydrophobic analyte of those tested (log $P_{o/w}$ value of ca. 3.5), there were some interactions with the PEGylated lipid coatings. Dichlorophenamide (log $P_{o/w}$ value of ca. 0.9), too, interacted fairly strongly with the phospholipid coatings. The separation results show that the higher the molar mass of the PEG lipid, the weaker are the interactions between small drugs and the membranes.

Interactions between PEGylated lipid aggregates and low-molar mass analytes were further studied with a mixture of nine FITC-labeled amino acids (LIF detection). The absolute migration times of the FITC-amino acids in coated capillaries were compared with data from an uncoated capillary. The best migration time repeatabilities (n=5) were seen with the POPC/PEG3000 dispersion (RSDs 0.3–1.5%) and with POPC/PEG2000 (RSDs 0.5–2.6%). However, with POPC/PEG1000 as coating the values were between 2.9 and 7.3%. With the uncoated capillary the migration times of the FITC-amino acids

decreased considerably from run to run (RSDs 3.4–6.3%). The corresponding electropherograms are shown in Figure 25 (modified from Figure 7 in paper V).

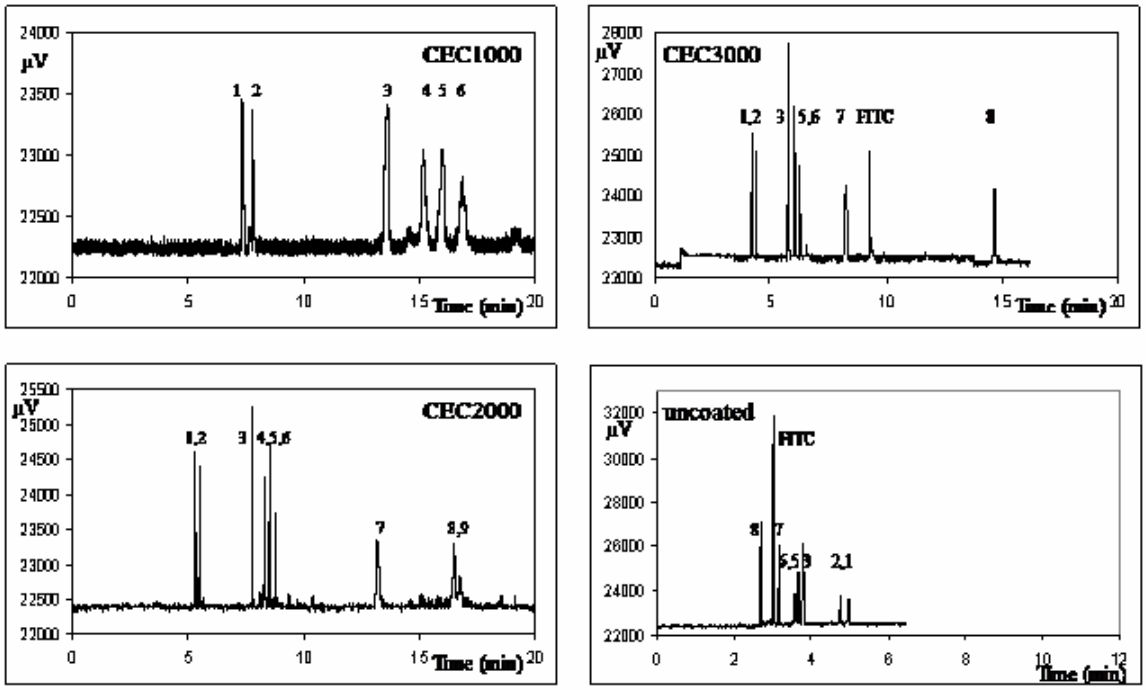


Figure 25. Electropherograms of FITC-labeled amino acids with lipid aggregates as coating material (CEC). Running conditions: BGE HEPES pH 7.4, ionic strength 9 mM (CEC); capillary 23.5/32 cm; voltage -30 kV (uncoated capillary +30 kV); temperature 25°C; sample injection 2 s 50 mbar; LIF detection at 488 nm. Peak numbering: FITC-labeled 1) aspartic acid, 2) glutamic acid, 3) alanine, 4) phenylalanine, 5) threonine, 6) tryptophan, 7) histidine, 8) arginine, 9) lysine.

The results revealed that the acyl chains of the PEG lipid influence the packing of the liposome membrane; the packing and shielding of charges were less effective with the C14 (DM) chains than the C18 (DS) chains.

6.2.7 Addition of cetyltrimethylammonium bromide in the coating

In an attempt to alter the surface charge of the POPC/DSPE-PEG3000 coating, we include the positively charged surfactant CTAB in the coating solution. Figure 26 presents EOF values during successive runs. As expected, the EOF was suppressed as the molar ratio of CTAB was increased. One interesting feature is seen in Figure 26; namely, CTAB has to be included in the coating solution before extrusion in order to achieve a stable coating. If CTAB is mixed into the coating solution after extrusion it is evidently not properly fixed in the bilayer and leaks out when runs are made.

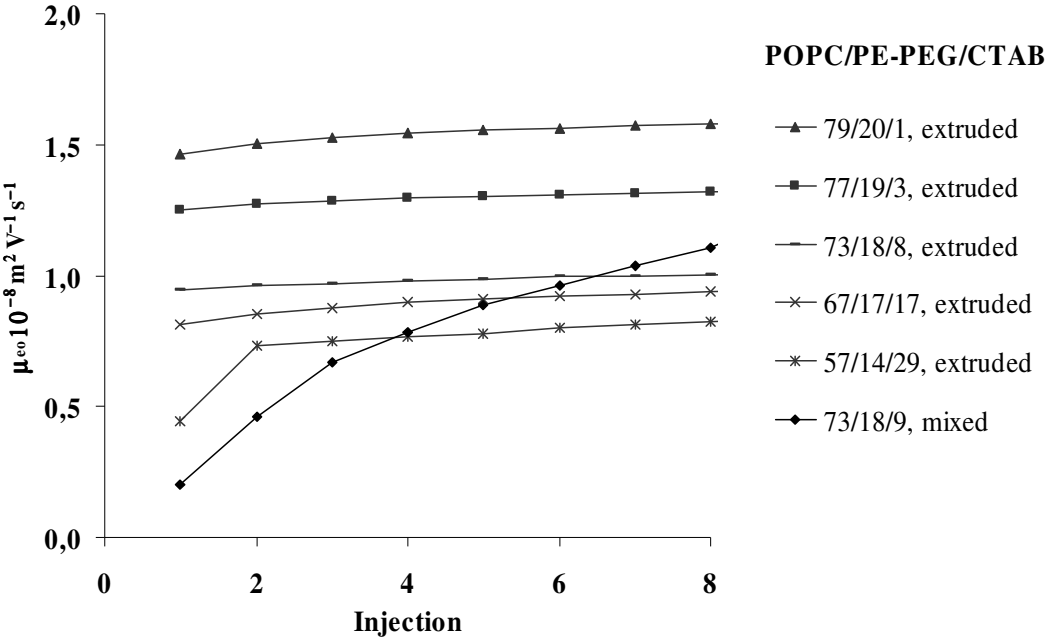


Figure 26. EOF values of POPC/DSPE-PEG3000 coatings with CTAB. Lipid solvent and BGE solution was HEPES pH 7.4. Running conditions: capillary 23.5/32 cm, voltage 20 kV, temperature 25°C, injection of EOF marker 2 s 50 mbar, UV detection at 200 nm.

6.3 Human red blood cell ghost lipid coating

After successful testing of liposome dispersions with different amounts of cholesterol, SM, and DPPC (see section 6.2.5), it was logical to move still further toward natural membranes. Since the bilayer of DPPC, SM, and cholesterol lacks the complex assembly of a cellular membrane, fused silica capillaries were next coated with human RBC ghost lipids. In a recent study on the main lipid components of human erythrocyte membrane [10], the cholesterol concentration was determined to be 54.0%, while the phospholipid concentrations of SM, PC, PS/phosphatidylinositol, and phosphatidylethanolamine were 12.1%, 16.7%, 6.4%, and 10.8%, respectively.

The results demonstrated that the capillary can be successfully coated with RBC ghost lipids. Two RBC liposome dispersions were prepared from the same stock solution, and a fresh capillary was coated at 25 °C with dispersions that were one day, one week, and two months old. In the case of the first RBC dispersion, the EOF was suppressed to 2.3–2.8 $\times 10^{-8}$ $m^2V^{-1}s^{-1}$ (average of six runs), and the relative standard deviations (RSD) were 1.0–3.7%. In measurements on the second RBC dispersion, the EOF varied between 2.8 and 3.5 $\times 10^{-8}$ $m^2V^{-1}s^{-1}$ and the RSDs between 1.1 and 2.6% over two months.

In addition, the stability of the RBC ghost lipid coating was studied at different temperatures (Figure 27, Figure 4 in paper IV). The six runs each at 25 °C, 37 °C, and 45 °C were made with the same capillary starting from the lowest temperature. Given the normal change of EOF with temperature and the slight increase in EOF due to the

previous runs at lower temperatures, it was clear from the suppression and stability of EOF at each temperature that the capillary was well coated with the RBC ghost lipids.

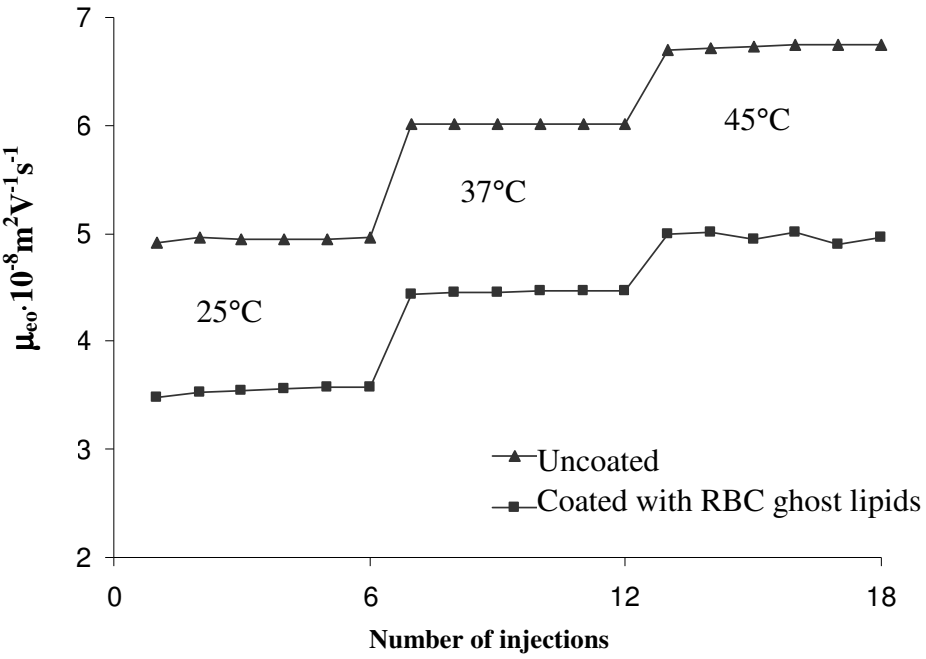


Figure 27. Change in EOF due to increasing temperature in a RBC ghost lipid-coated capillary (50.0/58.5 cm). Injection of 10% methanol for 5 s at 50 mbar, six separations each at 25 ºC, 37 ºC, and 45 ºC with a voltage of 20 kV, BGE 40 mM HEPES at pH 7.4, and detection at 200 nm.

RBC ghost lipids differ from the above artificial lipid mixture in that they contain a vast number of different phospholipids (variation in the head groups and fatty acid composition) [10]. The fused silica capillary was well coated with the RBC ghost lipids and the stability was good. The RBC ghost lipid coating was stable at the temperatures tested: 25 °C, 37 °C (physiological temperature), and 45 °C.

A group of steroids was also separated on a capillary coated with RBC ghost lipids. Figure 28 (Figure 5 in paper IV) displays the separation achieved in runs 1, 6, and 18 on a capillary coated with RBC ghost lipids. Although the model steroids (aldosterone, androstenedione, testosterone, 17-OH-progesterone, and progesterone) were not all baseline separated, there were hydrophobic interactions with the lipid phase, as is clearly seen for progesterone. The migration order of the steroids was determined by injecting each steroid separately. The separation was maintained in the 18th run, but the analysis time was prolonged. The EOF decreases as the negative charge of the coating decreases. Since the separation is maintained, the bilayer stability is still good, but some reorganization in the bilayer may have taken place leading to changes in the bilayer charge density or to coverage of the negative charges of the membrane lipids. The RBC ghost lipid extract may also contain small amounts of salts or protein residues, and the charge is affected when these are flushed out of the bilayer.

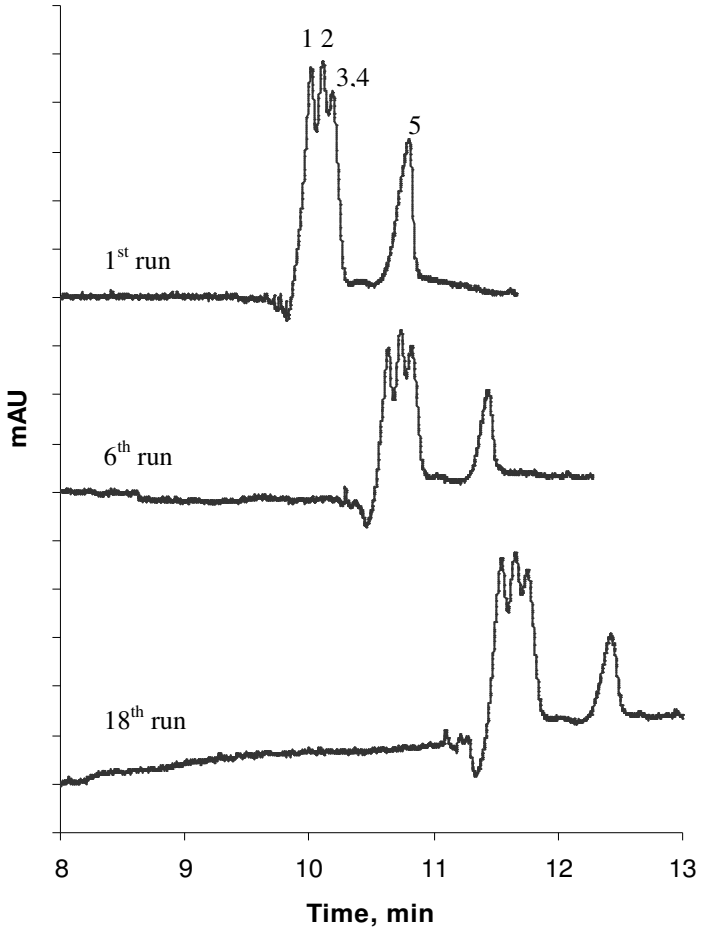


Figure 28. Comparison of the separation of steroids (1 aldosterone, 2 androstenedione, 3 testosterone, 4 17-OH-progesterone, and 5 progesterone in 5:95% (v/v) methanol/BGE) for the 1st, 6th, and 18th runs on a RBC ghost lipid-coated capillary (50.0/58.5 cm). Injection for 5 s at 50 mbar. Separation conditions: 20 kV, 25 ºC, 245 nm, BGE 40 mM HEPES at pH 7.4.

7 CONCLUSIONS

There is a growing interest in natural membranes and especially in membrane rafts and lipid–lipid interactions. An important focus has been the development of useful methods for lipid–protein and lipid–drug interactions. The stability of the coating and the easy coating procedure achieved in this work suggest that, in future, capillary electrophoresis could be used for a variety of lipid and membrane interaction studies. The study also demonstrates that cellular membranes (here from RBC) can be used for the separation of neutral steroids.

The phospholipid coating method is simple and rapid; it can be accomplished simply by flushing a silica capillary with liposomes for 10 minutes during a preconditioning step and allowing the capillary to stand, filled with the dispersion, for 15 minutes. The liposome composition plays an important role in the coating process. The storage of the capillaries affects the phospholipid coating: only capillaries filled with HEPES buffer solution during storage appeared to maintain their properties. Introduction of basic proteins and neutral steroids to the PC-coated capillaries showed that silanols on the capillary wall were almost completely shielded by the PC coating.

The effect of divalent metal ions (calcium, magnesium, and zinc) on phospholipid membranes was studied by coating a fused silica capillary with a liposome dispersion containing calcium, magnesium or zinc. All metal ions improved the stability of the PC-coated capillary. Increased rigidity, induced by metal ions immobilized into the membrane, is suggested to be the major reason for the improvement. Calcium appeared to improve the stability of the coating most.

Addition of CTAB as a stabilizer decreased the negative charge of the coating and suppressed the EOF further and hence also the interactions between proteins and the capillary wall. The stability of the coating was not improved, however. Increase in the concentration of CTAB in the coating solution resulted in prolongation of the analysis time as well as a decrease in coating stability. Addition of $CaCl_2$ to the coating solution as well (3:1 molar ratio of eggPC) improved the stability of the eggPC/CTAB coating markedly. Addition of $CaCl_2$ improved the reproducibility of the separation and improved the signal-to-noise ratio and plate numbers for lysozyme and α-chymotrypsinogen A. Prolongation of the coating time improved the signal-to-noise ratio and plate numbers for ribonuclease A and α-chymotrypsinogen A, but it had little effect on the peak of lysozyme.

Data on sizes and the zeta potentials of lipid aggregates showed that the higher the molar mass of the PEG lipid, the smaller were the particle sizes and the better was the shielding of the lipid membrane. This was evident in AFM and EM data, which showed the presence of discoidal micelles (disks) in the POPC/DSPE-PEG3000 dispersion, both liposomes and disks in the POPC/DSPE-PEG2000 dispersion, and mostly liposomes in the POPC/DSPEG1000 dispersion. The dependency of the zeta potentials and sizes of the lipid aggregates on the packing of the aggregates on silica surfaces was evaluated by AFM and EM, with a QCM and by EOF measurements in CEC.

From the electrophoretic stability measurements, it is clear that PC is an important component of the coating solution if a good and stable coating is to be achieved. DPPC alone can form a good coating. The bilayer formation is not disrupted, however, when more natural membranes are prepared by including up to 30 mol% of cholesterol and SM. Coating solutions containing 70-100 mol% of DPPC and 30 to 0 mol% of cholesterol and SM give good coatings.

The effect of the temperature-induced gel- to fluid-state transition on the coating stability was investigated by coating a fused silica capillary with DPPC above and below the T_m of the liposome and carrying out calorimetric measurements. Working in the gel state significantly increased the stability of the coating, confirming that the rigidity of the membrane is essential in improving the coating stability. The results from the calorimetric studies are in good accord with the results published by other groups. Adding cholesterol to a liposome dispersion of DPPC or DPPC and SM broadens the main phase transition and eventually eliminates it at higher (> 30 mol%) cholesterol concentrations. Addition of cholesterol to a fluid DPPC coating makes the coating more gel-like and more stable by increasing the orientation of the phospholipids. In addition, RBC ghost lipids can be successfully coated on fused silica capillaries. The ghost lipid coating is stable at 25, 37, and 45 °C.

The findings of the work show that the zwitterionic liposomes must be gel-like and rigid in order to achieve a stable coating on a fused silica capillary. The stability of the coating and the easy coating procedure promise to give the method important applications in analyte–membrane interaction studies — for example, studies on the interaction of drugs and biomolecules with natural membranes. Connection of the coated capillary to a mass spectrometer, gives a promising set-up for studies analyte–membrane interaction.

8 REFERENCES

[1] Bangham, A. D., *Hospital practice Dec. 15th* (1992) 51-61. Liposomes: realizing their promise

[2] Duffy, C. F., Gafoor, S., Richards D. P. Admadzadeh, H., O'Kennedy, R., Arriaga E. A., *Anal. Chem. 74* (2001) 1855-1861. Determination of properties of individual liposomes by capillary electrophoresis with postcolumn laser-induced fluorescence detection

[3] Manetto, G., Bellini, M. S., Deyl, Z., *J. Chromatogr. A 990* (2003) 205-214. Application of capillaries with minimized electroosmotic flow to the electrokinetic study of acidic drug–β-oleyl-γ-palmitoyl-L-α-phosphatidyl choline liposome interactions

[4] Colletier, J. P., Chaize, B., Winterhalter, M., Fournier, D., *BMC Biotechnol. 2* (2002) 9-16. Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer

[5] Abraham, S. A., Waterhouse, D. N., Mayer, L. D., Cullis, P. R., Madden, T. D., Bally, M. B., *Methods Enzymol. 391* (2005) 71-97. The liposomal formulation of doxorubicin

[6] Ng, A. W. K., Wasan, K. M., Lopez-Berestein, G., *Methods Enzymol. 391* (2005) 304-313. Liposomal polyene antibiotics

[7] Hac-Wydro, K., Dynarowicz-Latka, P., Grybowska, J., Borowski, E., *Colloids Surf. B 46* (2005) 7-19. How does the N-acylation and esterification of amphotericin B molecule affect its interactions with cellular membrane components—the Langmuir monolayer study

[8] New, R. R. C. (Editor), *Liposomes: a practical approach*. Oxford University Press, New York 1990, p. 1

[9] Huang, C., Li, S., *Biochim. Biophys. Acta 1422* (1999) 273-307. Calorimetric and molecular mechanics studies of the thermotropic phase behaviour of membrane phospholipids

[10] Koumanov, K. S., Tessier, C., Momchilova, A. B., Rainteau, D., Wolf, C., Quinn, P.J., *Arch. Biochem. Biophys. 434* (2005) 150-158. Comparative lipid analysis and structure of detergent-resistant membrane raft fractions isolated from human and ruminant erythrocytes

[11] Rothman, J. E., Lenard, J., *Science 195* (1977) 743-753. Membrane asymmetry

[12] Bloch, K., *Cholesterol: evolution of structure and function* in Vance D. E., Vance, J. E. (Editors), *Biochemistry of lipids, lipoproteins and membranes*. Elsevier, Amsterdam 1991, pp. 363-381.

[13] Ladbrooke, B. D., Williams, R. M., Chapman, D., *Biochim. Biophys. Acta 150* (1968) 333-340. Studies on lecithin–cholesterol–water interactions by differential scanning calorimetry and X-ray diffraction

[14] Huang, J., Buboltz, J. T., Feigenson, G. W., *Biochim. Biophys. Acta 1417* (1999) 89-100. Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers

[15] Huang, J., Feigenson, G. W., *Biophys. J. 76* (1999) 2142-2157. A microscopic interaction model of maximum solubility of cholesterol in lipid bilayers

[16] Bloom, M., Mouritsen, O. G., *Can. J. Chem. 66* (1988) 706-712. The evolution of membranes
[17] Yeagle, P. L., *Biochim. Biophys. Acta 822* (1985) 267-287. Cholesterol and the cell membrane
[18] McMullen, T. P. W., Lewis, R. N. A., McElhaney, R. N., *Biochemistry 32* (1993) 516-522. Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behaviour of a homologous series of linear saturated phosphatidylcholines
[19] Slotte, J. P., *Chem. Phys. Lip. 102* (1999) 13-27. Sphingomyelin–cholesterol interactions in biological and model membranes
[20] Simons, K., Ikonen, E., *Nature 387* (1997) 569-572. Functional rafts in cell membranes
[21] Silvius, J. R., *Biochim. Biophys. Acta 1610* (2003) 174-183. Role of cholesterol in lipid raft formation: lessons from lipid model systems
[22] Ohvo-Rekilä, H., Ramstedt, B., Leppimäki, P., Slotte, J. P., *Progr. Lip. Research 41* (2002) 66-97. Cholesterol interactions with phospholipids in membranes
[23] Brown, R. E., *J. Cell. Sci. 111* (1998) 1-9. Sphingolipid organization in biomembranes: what physical studies of model membranes reveal
[24] Holopainen, J. M., Metso, A. J., Mattila, J.-P., Jutila, A., Kinnunen, P. K. J., *Biophys. J. 86* (2004) 1510-1520. Evidence for the lack of a specific interaction between cholesterol and sphingomyelin
[25] Brown, D. A., London, E., *J. Biol. Chem. 275* (2000) 17221-17224. Structure and function of sphingolipid- and cholesterol-rich membrane rafts
[26] Edidin, M., *Annu. Rev. Biophys. Biomol. Struct. 32* (2003) 257-283. The state of lipid rafts: from model membranes to cells
[27] Guo, W., Kurze, V., Huber, T., Afdhal, N. H., Beyer, K., Hamilton, J.A, *Biophys. J. 83* (2002) 1465-1478. A solid-state NMR study of phospholipid–cholesterol interactions: sphingomyelin–cholesterol binary systems
[28] Puu, G., Gustafson, I., *Biochim. Biophys. Acta 1327* (1997) 149-161. Planar lipid bilayers on solid supports from liposomes – factors of importance for kinetics and stability
[29] Wilschut, J., Hoekstra, D., *Trends Biochem. Sci. 9* (1984) 479-483. Membrane fusion: from liposomes to biological membranes
[30] Leckband, D. E., Helm, C. A., Israelachvili, J., *Biochemistry 32* (1993) 1127-1140. Role of calcium in the adhesion and fusion of bilayers
[31] Hautala, J. T., Wiedmer, S. K., Riekkola, M.-L., *Anal. Bioanal. Chem. 378* (2004) 1769-1776. Anionic liposomes in capillary electrophoresis: effect of calcium on 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine/phosphatidylserine coatings in silica capillaries
[32] Hincha, D. K., *Biochim. Biophys. Acta 1611* (2003) 180-186. Effects of calcium-induced aggregation on the physical stability of liposomes containing plant glycolipids
[33] Garidel, P., Blume, A., Hübner, W., *Biochim. Biophys. Acta 1466* (2000) 245-259. A Fourier transform infrared spectroscopic study of the interaction of alkaline

earth cations with the negatively charged phospholipid 1.2-dimyristoyl-*sn*-glycero-3-phosphoglycerol

[34] Hautala, J. T., Riekkola, M.-L., Wiedmer, S. K., *J. Chromatogr. A 1150* (2007) 339-347. Anionic phospholipid coatings in capillary electrochromatography Binding of Ca^{2+} to phospholipid phosphate group

[35] Böckmann, R. A., Grubmüller, H., *Angew. Chem. 243* (2004) 1021-1024. Multistep binding of divalent cations to phospholipid bilayers; a molecular dynamics study

[36] Binder, H., Zschörnig, O., *Chem. Phys. Lipids 115* (2002) 39-61. The effect of metal cations on the phase behaviour and hydration characteristics of phospholipid membranes

[37] Binder, H., Arnold, K., Ulrich, A. S., Zschörnig, O., *Biophys. Chem. 90* (2001) 57-74. Interaction of Zn^{2+} with phospholipid membranes

[38] Hu, W., Haddad, P. R., Hasebe, K., Tanaka, K., *Anal. Commun. 36* (1999) 97-100. Electrostatic ion chromatography of cations using an N-dodecylphosphocholine zwitterionic stationary phase and water as the mobile phase

[39] Reviakine, I., Simon, A., Brisson, A., *Langmuir 16* (2000) 1473-1477. Effect of Ca^{2+} on the morphology of mixed DPPC–DOPS supported phospholipid bilayers

[40] Isomaa, B., *Biochemical Pharmacology 28* (1979) 975-980. Interactions of surface-active alkyltrimethylammonium salts with the erythrocyte membrane

[41] Strauss, G., Alhaique, F., Memoli, A., Santucci, E., Riccieri, F. M., *Polymer Preprints 27* (1986) 48. The stability of drug-carrying liposomes in the presence of detergents

[42] Ottiger, M., Bax, A., *J. Biomolecular NMR 13* (1999) 187-191. Bicelle-based liquid crystals for NMR-measurement of dipolar couplings at acidic and basic pH values

[43] de la Maza, A., Parra, J. L., *J. Controlled Release 37* (1995) 33-42. Solubilization of unilamellar liposomes caused by quaternary ammonium surfactants

[44] Sujatha, J., Mishra, A. K., *J. Photochem. Photobiol. A: Chem. 104* (1997) 173-178. Effect of ionic and neutral surfactants on the properties of phospholipid vesicles: investigation using fluorescent probes

[45] Eytan, G. D., Mary, T., Broza, R., Shaltin, Y., *Biochim. Biophys. Acta 778* (1984) 38-48 Cationic amphiphiles induce fusion of acidic liposomes

[46] Urbaneja, M. A., Alonso, A., Gonzales-Manas, J. M., Goni, F. M., Partearroyo, M. A., Tribout, M., Paredes, S., *Biochem. J. 270* (1990) 305-308. Detergent solubilization of phospholipid vesicles

[47] Tsao, H.-K., Tseng, W. L., *J. Chemical Physics 115* (2001) 8125-8132. The interactions between ionic surfactants and phosphatidylcholine vesicles: conductometry

[48] Lasic, D. D., Belic, A., Valentincic, T., *J. Am. Chem. Soc. 110* (1988) 970-971. A new method for the instant preparation of large unilamellar vesicles

[49] Artyukhin, A. B., Stroeve, P., *Ind. Eng. Chem. Res. 42* (2003) 2156-2162. Effects of corrosive chemicals on solid-supported lipid bilayers as measured by surface plasmon resonance

[50] Blume, G., Cevc, G., *Biochim. Biophys. Acta 1029* (1990) 91-97. Liposomes for the sustained drug release in vivo
[51] Woodle, M. C., Lasic, D. D., *Biochim. Biophys. Acta 1113* (1992) 171-199, Sterically stabilized liposomes.
[52] Allen, T. M., *Trends Pharmacol Sci. 15* (1994) 215-220. Long-circulating (sterically stabilized) liposomes for targeted drug delivery
[53] Qiu, L., Yan, B., You, H., *Pharm. Res. 23* (2006) 1-30. Polymer architecture and drug delivery
[54] Silvander, M. Hansson, P. Edwards, K., *Langmuir 16* (2000) 3696-3702. Liposomal surface potential and bilayer packing as affected by PEG-lipid inclusion
[55] Belsito, S., Bartucci, R., Sportelli, L., *Biophys. Chem. 75* (1998) 33-43. Sterically stabilized liposomes of DPPC/DPPE-PEG:2000. A spin label ESR and spectrophotometric study
[56] Yang, Q., Lundahl, P., *Biochemistry 34* (1995) 7289-7292. Immobilized proteoliposome affinity chromatography for quantitative analysis of specific interactions between solutes and membrane proteins. Interaction of cytochalasin B and D-glucose with the glycose transporter glut1
[57] Brekkan, E., Lundqvist, A., Lundahl, P., *Biochemistry 35* (1996) 12141-12145. Immobilized membrane vesicle or proteoliposome affinity chromatography. Frontal analysis of interactions of cytochalasin B and D-glucose with the human red cell glucose transporter
[58] Boija, E., Johansson, G., *Biochim. Biophys. Acta 1758* (2006) 620-626. Interactions between model membranes and lignin-related compounds studied by immobilized liposome chromatography
[59] Tsirkin, I., Grushka, E., *J. Chromatogr. A 919* (2001) 245-254. Characterization of dynamically prepared phospholipids-modified reversed-phase columns
[60] Liu, X. Y., Nakamura, Q., Kamo, N., Miyake, J., *J. Chromatogr. A 961* (2002) 113-118. Immobilized liposome chromatography to study drug–membrane interactions
[61] Liu, X.-Y., Nakamura, C., Nakamura, N., Hirano, T., Shinbo, T., Miyake J., *J. Chromatogr. A 1087* (2005) 229-235. Detection of polychlorinated biphenyls using an antibody column in tandem with a fluorescent liposome column. Effect of albumin on phospholipase A_2-catalyzed membrane leakage
[62] Liu, X.-Y., Nakamura, C., Tanimoto, I., Miyake, S., Nakamura, N., Hirano, T., Miyake J., *Anal. Chim. Acta 578* (2006) 43-49. High sensitivity detection of bisphenol A using liposome chromatography
[63] Yoshimoto, N., Yoshimoto, M., Yasuhara, K., Shimanouchi, T., Umakoshi, H, Kuboi, R., *Biochem. Eng. J. 29* (2006) 174-181. Evaluation of temperature and guanidine hydrochloride-induced protein–liposome interactions by using immobilized liposome chromatography
[64] Yang, C. Y., Cai, S. J., Liu, H., Pidgeon, C., *Adv. Drug Delivery Rev. 23* (1996) 229-256. Immobilized artificial membranes — screen for drug membrane interactions

[65] Krause, E., Dathe, M., Wieprecht, T., Bienert, M., *J. Chromatogr. A 849* (1999) 125-133. Noncovalent immobilized artificial membrane chromatography, an improved method for describing peptide–lipid bilayer interactions

[66] Hanna, M., de Biasi, V., Bond, B., Camilleri, P., Hutt, A. J., *Chromatographia 52* (2000) 710-720. Biomembrane lipids as components of chromatographic phases: comparative chromatography on coated and bonded phases

[67] Mao, X., Kong, L., Luo, Q., Li, X., Zou, H., *J. Chromatogr. A 779* (2002) 331-339. Screening and analysis of permeable compounds in *Radix Angelica Sinensis* with immobilized liposome chromatography

[68] Hu, W., Haddad, P. R., Tanaka, K., Mori, M., Tekura, K., Hasebe, K., Ohno, M., Kamo, N., *J. Chromatogr. A 997* (2003) 237-242. Creation and characteristics of phosphatidylcholine stationary phases for the chromatographic separation of inorganic anions

[69] Zhang, W.-N. Hu, Z.-X., Liu, Y., Feng, Y.-Q., Da, S.-L., *Talanta 67* (2005) 1023-1028. Study in interaction between drug and membrane by using liposome coated zirconia–magnesia chromatography

[70] Wiedmer, S. K., Jussila, M. S., Riekkola, M.-L., *Trends Anal. Chem. 23* (2004) 562-582. Phospholipids and liposomes in liquid chromatographic and capillary electromigration techniques

[71] Owen, R. L., Strasters, J. K., Breyer; E. D., *Electrophoresis 26* (2005) 735-751. Lipid vesicles in capillary electrophoretic techniques: characterization of structural properties and associated membrane-molecule interactions

[72] Gómez-Hens, A., Fernández-Romero, J. M., *Trends Anal. Chem. 24* (2005) 9-19. The role of liposomes in analytical processes

[73] Gómez-Hens, A., Fernández-Romero, J. M., *Trends Anal. Chem. 25* (2006) 167-178. Analytical methods for the control of liposomal delivery systems

[74] Bilek, G., Kremser, L., Blaas, D., and Kenndler, E., *J. Chromatogr. B 841* (2006) 38-51. Analysis of liposomes by capillary electrophoresis and their use as carriers in electrokinetic chromatography

[75] Zhang, Y., Zhang, R., Hjerten, S., Lundahl, P., *Electrophoresis 16* (1995) 1519-1523. Liposome capillary electrophoresis for analysis of interactions between lipid bilayers and solutes

[76] Wiedmer, S. K., Jussila, M. S., Holopainen, J. M., Alakoskela, J.-M., Kinnunen, P. K. J., Riekkola, M.-L., *J. Sep. Sci. 25* (2002) 427-437. Cholesterol-containing phosphatidylcholine liposomes: characterization and use as dispersed phase in electrokinetic capillary chromatography

[77] Wiedmer, S. K., Holopainen, J. M., Mustakangas, P., Kinnunen, P. K. J., Riekkola, M.-L., *Electrophoresis 21* (2000) 3191-3198. Liposomes as carriers in electrokinetic capillary chromatography

[78] Wiedmer, S. K., Hautala, J., Holopainen, J. M., Kinnunen, P. K. J., Riekkola, M.-L., *Electrophoresis 22* (2001) 1305-1313. Study on liposomes by capillary electrophoresis

[79] Burns, S. T., Khaledi, M. G., *J. Pharm. Sci. 91* (2002) 1601-1612. Rapid determination of liposome–water partition coefficients (K_{lw}) using liposome electrokinetic chromatography (LEKC)

[80] Burns, S. T., Agbodjan, A. A., Khaledi, M. G., *J. Chromatogr. A 973* (2002) 167-176. Characterization of solvation properties of lipid bilayer membranes in liposome electrokinetic chromatography

[81] Pascoe, R. J., Masucci, J. A., Foley, J. P., *Electrophoresis 27* (2006) 793-804. Investigation of vesicle electrokinetic chromatography as an *in vitro* assay for the estimation of intestinal permeability of pharmaceutical drug candidates

[82] Carrozzino; J. M., Khaledi, M. G., *J. Chromatogr. A 1079* (2005) 307-316. pH effects on drug interactions with lipid bilayers by liposome electrokinetic chromatography

[83] Yang, Q., Liu, X.-Y. Miyake, J., Toyotama, H., *Supramol. Sci. 5* (1998) 769-772. Self-assembly and immobilization of liposomes in fused-silica capillary by avidin–biotin binding

[84] Cunliffe, J. M., Baryla, N. E., Lucy, C. A., *Anal. Chem. 74* (2002) 776-783. Phospholipid bilayer coatings for the separation of proteins in capillary electrophoresis

[85] Örnskov, E., Ullsten, S., Söderberg, L., Markides, K. E., Folestad, S., *Electrophoresis 23* (2002) 3381-3384. Method for immobilization of liposomes in capillary electrophoresis by electrostatic interaction with derivatized agarose

[86] Manetto, G., Bellini, M. S., Deyl, Z., *J. Chromatogr. A 990* (2003) 281-289. Affinity electrochromatography of acidic drugs using a liposome-modified capillary

[87] McKeon, J., Khaledi, M. G., *J. Chromatogr. A 1004* (2003) 39-46. Evaluation of liposomal delivery of antisense oligonucleotide by capillary electrophoresis with laser-induced fluorescence detection

[88] Bo, T., Wiedmer, S. K., Riekkola, M.-L., *Electrophoresis 25* (2004) 1784-1791. Phospholipid–lysozyme coating for chiral separation in capillary electrophoresis

[89] Corradini, D., Mancini, G., Bello, C., *J. Chromatogr. A 1051* (2004) 103-110. Use of liposomes as a dispersed pseudo-stationary phase in capillary electrophoresis of basic proteins

[90] Hautala, J. T., Wiedmer, S. K., Riekkola, M.-L., *Electrophoresis 26* (2005) 176-186. Influence of pH on formation and stability of phosphatidylcholine/ phosphatidylserine coatings in fused-silica capillaries

[91] Wang, C., Lucy, C. A., *Anal. Chem. 77* (2005) 2015-2021. Oligomerized phospholipid bilayers as semipermanent coatings in capillary electrophoresis

[92] Nesbitt, C. A., Lo, J. T.-M., Yeung, K. K.-C., *J. Chromatogr. A 1073* (2005) 175-180. Over 1000-fold protein preconcentration for microliter-volume samples at a pH junction using capillary electrophoresis

[93] Varjo, S. J. O., Hautala, J. T., Wiedmer, S. K., Riekkola, M.-L., *J. Chromatogr. A 1081* (2005) 92-98. Small diamines as modifiers for phophatidylcholine/ phosphatidylserine coatings in capillary electrochromatography

[94] Wiedmer, S. K., Jussila, M., Hakala, R. M. S., Pystynen, K.-H., Riekkola, M.-L., *Electrophoresis 26* (2005) 1920-1927. Piperazine-based buffers for liposome coating of capillaries for electrophoresis

[95] Ross, E. E., Mansfield, E., Huang, Y., Aspinwall, C. A., *J. Am. Chem. Soc. 127* (2005) 16756-16757. *In situ* fabrication of three-dimensional chemical patterns in fused silica separation capillaries with polymerized phospholipids

[96] Han, N.-Y., Hautala, J. T., Bo, T., Wiedmer, S. K., Riekkola, M.-L., *Electrophoresis 27* (2006) 1502-1509. Immobilization of phospholipid-avidin on fused-silica capillaries for chiral separation in open-tubular capillary electrochromatography

[97] Yohannes, G., Pystynen, K.-H., Riekkola, M.-L., Wiedmer, S. K., *Anal. Chim. Acta 560 (*2006) 50-56. Stability of phospholipid vesicles studied by asymmetrical flow field-flow fractionation and capillary electrophoresis

[98] Falck, E., Hautala, J. T., Karttunen, M. Kinnunen, P. K. J., Patra, M., Saaren-Seppälä, H., Vattulainen, I., Wiedmer, S. K., Holopainen, J. M., *Biophys J. 91* (2006) 1787-1799. Interaction of fusidic acid with lipid membranes: implications to the mechanism of antibiotic activity

[99] Mansfield, E., Ross, E. E., Aspinwall, G. A., *Anal. Chem. 79* (2007) 3135-3141. Preparation and characterization of gross-linked phospholipid bilayer capillary coatings for protein separations

[100] White, C. M., Luo, R., Archer-Hartmann, S. A., Holland, L. A., *Electrophoresis 28* (2007) 3049-3055. Electrophoretic screening of ligands under suppressed EOF with an inert phospholipid coating

[101] Muhonen, J., Vidgren, J., Helle, A., Yohannes, G., Viitala, T., Holopainen, J. M., Wiedmer, S. K., *Anal. Biochem. 374* (2008) 133-142. Interactions of fusidic acid and elongation factor G with lipid membranes

[102] Wiedmer, S. K., Bo, T., Riekkola, M.-L., *Anal. Biochem. 373* (2008) 26-33. Phospholipid–protein coatings for chiral capillary electrochromatography

[103] Mei, J., Xu, J.-R., Zhang, Q.-R., Feng, Y.-Q., *Talanta 75* (2008) 104-110. Immobilized phospholipid capillary electrophoresis for study of drug–membrane interactions and prediction of drug activity

[104] Gulcev, M. D., Lucy, C. A., *Anal. Chem. 80* (2008) 1806-1812. Factors affecting the behaviour and effectiveness of phospholipid bilayer coatings for capillary electrophoretic separations of basic proteins

[105] Söderlund, T., Lehtonen, J. Y. A., Kinnunen, P. K. J., *Mol. Pharmacol. 55* (1999) 32-38. Interactions of cyclosporine A with phospholipid membranes: effect of cholesterol

[106] Jutila, A., Kinnunen, P. K. J., *J. Phys. Chem. B 101* (1997) 7635-7640. Novel features of the main transition of dimyristoylphosphocholine bilayers revealed by fluorescence spectroscopy

[107] Rapuano, R., Carmona-Ribeiro, A. M., *J. Colloid Interface Sci. 193* (1997) 104-111. Physical adsorption of bilayer membranes on silica

[108] Leonenko, Z. V., Carnini, A., Cramb, D. T., *Biochim. Biophys. Acta 1509* (2000) 131-147. Supported planar bilayer formation by vesicle fusion: the interaction of phospholipid vesicles with surfaces and the effect of gramicidin on bilayer properties using atomic force microscopy

[109] Reviakine, I., Brisson, A., *Langmuir 16* (2000) 1806-1815. Formation of supported phospholipid bilayers from unilamellar vesicles investigated by atomic force microscopy
[110] Jass, J., Tjärnhage, T., Puu, G., *Biophys. J. 79* (2000) 3153-3163. From liposomes to supported, planar bilayer structures on hydrophilic and hydrophobic surfaces: an atomic force microscopy study
[111] Viitala, T., Hautala, J. T., Vuorinen, J., Wiedmer, S. K., *Langmuir 23* (2007) 609-618. Structure of anionic phospholipid coatings on silica by dissipative quartz crystal microbalance
[112] Wahlund, K.-G., Litzén, A., *J. Chromatogr. 461* (1989) 73-87. Application of an asymmetrical flow field-flow fractionation channel to the separation and characterization of proteins, plasmids, plasmid fragments, polysaccharides and unicellular algae
[113] Wahlund, K.-G., Giddings, J. C., *Anal. Chem. 59* (1987) 1332-1339. Properties of an asymmetrical flow field-flow fractionation channel having one permeable wall
[114] Litzén, A., Wahlund, K.-G., *J. Chromatogr. 476* (1989) 413-421. Improved separation speed and efficiency for proteins, nucleic acids and viruses in asymmetrical flow field flow fractionation
[115] Litzén, A., Wahlund, K.-G., *Anal. Chem. 63* (1991) 1001-1007. Zone broadening and dilution in rectangular and trapezoidal asymmetrical flow field-flow fractionation channels
[116] Dubochet, J., Adrian, M., Chang, J. J. Homo, J. C. Lepault, J. McDowall, A. W. Schultz, P., *Quart. Rev. Biophys. 21* (1988) 129-228. Cryo-electron microscopy of vitrified specimens
[117] Sauerbrey, G., *Z. Phys. 155* (1959) 206-222. The use of quartz oscillators for weighing thin layers and for microweighing
[118] Bandey, H. L., Martin, S. J., Cernosek, R. W., Hillman, A. R., *Anal. Chem. 71* (1999) 2205-2214. Modeling the responses of thickness-shear mode resonators under various loading conditions
[119] Melanson, J. E., Baryla, N. E., Lucy, C. A., *Anal. Chem. 72* (2000) 4110-4114. Double-chained surfactants for semipermanent wall coatings in capillary electrophoresis
[120] Jones, M. N., *Adv. Coll. Interface Sci. 54* (1995) 93-128. The surface properties of phospholipid liposome systems and their characterisation
[121] Radko, S. P., Crambach, A., *Electrophoresis 23* (2002)1957-1972. Separation and characterization of sub-µm- and µm-sized particles by capillary zone electrophoresis
[122] Nollert, P., Kiefer, H., Jaehnig, F., *Biophys. J. 69* (1995) 1447-1455. Lipid vesicle adsoption versus formation of planar bilayers on solid surfaces
[123] Träuble, H., Eibl, H., *Proc. Natl. Acad. Sci. U. S. A. 71* (1974) 214-219. Electrostatic effects on lipid phase transitions: membrane structure and ionic environment
[124] Kastner, M. (Editor), *Protein liquid chromatography*, Elsevier, Amsterdam 2000, chapter 6

[125] Malmsten, M., *Coll. Int. Sci. 172* (1995) 106-115. Protein adsorption at phospholipid surfaces
[126] Holmlin, R. E., Chen, X., Chapman, R. G., Takayama, S., Whitesides, G. M., *Langmuir 17* (2001) 2841-2850. Zwitterionic SAMs that resist non-specific adsorption of protein from aqueous buffer
[127] Diress, A. G., Lucy, C. A., *J. Chromatogr. A 1027* (2004) 185-191. Electroosmotic flow reversal for the determination of inorganic anions by capillary electrophoresis with methanol–water buffers
[128] Beckers, J. L., Bocek, P., *Electrophoresis 23* (2002) 1947-1952. Multiple effect of surfactants used as additives in background electrolytes in capillary zone electrophoresis: cetyltrimethylammonium bromide as example of model surfactant
[129] Taylor, K. M. G., Morris, R. M., *Thermochim. Acta 248* (1995) 289-301. Thermal analysis of phase transition behaviour in liposomes
[130] Ladbrooke, B. D., Chapman, D., *Chem. Phys. Lipids 3* (1969) 304-367. Thermal analysis of lipids, proteins and biological membranes. A review and summary of some recent studies
[131] Pabst, G., Katsaras, J., Raghunathan, V. A., Rappolt, M., *Langmuir 19* (2003) 1716-1722. Structure and interactions in the anomalous swelling regime of phospholipid bilayers
[132] Snyder, B., Freire, E., *Proc. Natl. Acad. Sci. U.S.A. 77* (1980) 4055-4059. Compositional domain structure in phosphatidylcholine–cholesterol and sphingomyelin–cholesterol bilayers
[133] Huang, T.-H., Lee, C. W. B., Das Gupta, S. K., Blume, A., Griffin, R. G., *Biochemistry 32* (1993) 13277-13287. A ^{13}C and ^{2}H nuclear magnetic resonance study of phosphatidylcholine/cholesterol interactions: characterization of liquid–gel phases
[134] Ipsen, J. H., Karlström, G., Mouritsen, O. G., Wennerström, H., Zuckermann, M. H., *Biochim. Biophys. Acta 905* (1987) 162-172. Phase equilibria in the phosphatidylcholine-cholesterol system
[135] Vist, M. R., Davis, J. H., *Biochemistry 29* (1990) 451-464. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^{2}H nuclear magnetic resonance and differential scanning calorimetry
[136] Anderson, T. G., McConnell, H. M., *Biophys. J. 81* (2001) 2774-2785. Condensed complexes and the calorimetry of cholesterol-phospholipid bilayers
[137] Spink, C. H., Manley, S., Breed, M., *Biochim. Biophys. Acta 1279* (1996) 190-196. Thermodynamics of transfer of cholesterol from gel to fluid phases of phospholipid bilayers
[138] Mouritsen, O. G., Zuckermann, M. J., *Lipids 39* (2004) 1101-1113. What's so special about cholesterol?
[139] Edidin, M., *Nature 4* (2003) 414-418. Lipids on the frontier: a century of cell-membrane bilayers
[140] Sankaram, M. B., Thompson, T. E., *Biochemistry 29* (1990) 10670-10675. Interaction of cholesterol with various glycerophospholipids and sphingomyelin

[141] Xu, X., London, E., *Biochemistry 39* (2000) 843-849. The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation
[142] Simons, K., Vaz, W. L. C., *Annu. Rev. Biophys. Biomol. Struct. 33* (2004) 269-295. Model systems, lipid rafts, and cell membranes
[143] de Almeida R. F. M., Fedorov, A., Prieto, M., *Biophys. J. 85* (2003) 2406-2416. Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts
[144] Ashok, B., Arleth, L., Hjelm, R. P., Rubinstein, I., Önyüksel, H., *J. Pharm. Sci. 93* (2004) 2476-2487. *In vitro* characterization of PEGylated phospholipid micelles for improved drug solubilization: effects of PEG chain length and PC incorporation
[145] Sandström, M. C. Johansson, E. Edwards, K., *Langmuir 23* (2007) 4192-4198. Structure of mixed micelles formed in PEG-lipid/lipid dispersions
[146] Yoshida, A. Hashizaki, K. Yamauchi, H. Sakai, H. Yokoyama, S., Abe M., *Langmuir 15* (1999) 2333-2337. Effect of lipid with covalently attached poly(ethylene glycol) on the surface properties of liposomal bilayer membranes
[147] Garbuzenko, O., Zalipsky, S., Quazen, M., Barenholz Y., *Langmuir 21* (2005) 2560-2568. Electrostatics of PEGylated micelles and liposomes containing charged and neutral lipopolymers
[148] Silvander, M., *Progr. Colloid Polym. Sci. 120* (2002) 35-40. Steric stabilization of liposomes – a review
[149] Woodle, M. C., *Adv. Drug Delivery Rev. 16* (1995) 249-265. Sterically stabilized liposome therapeutics
[150] Johnsson, M., Edwards, K., *Biophys. J. 85* (2003) 3839-3847. Liposomes, disks, and spherical micelles: aggregate structure in mixtures of gel phase phosphatidylcholines and poly(ethylene glycol)-phospholipids
[151] Edwards, K., Johnsson, M., Karlsson, G., Silvander M., *Biophys. J. 73* (1997) 258-266. Effect of polyethyleneglycol-phospholipids on aggregate structure in preparations of small unilamellar liposomes
[152] Ueno, M., Sriwongsitanont, S., *Polymer 46* (2005) 1257-1267. Effect of PEG lipid on fusion and fission of phospholipid vesicles in the process of freeze-thawing